PATENT APPLICATION

PATENT AND TRADEMARK OFFICE

BEFORE THE HONORABLE BOARD OF PATENT APPEALS AND INTERFERENCES

In re the Application of

Didier RAOULT et al.

On Appeal from Group: 1645

Application No.: 09/936,921

Examiner: P. BASKAR

Filed: September 24, 2001

Docket No.: 110530

For: DIAGNOSIS OF WHIPPLE'S DISEASE

APPEAL BRIEF TRANSMITTAL

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Attached hereto is our Brief on Appeal in the above-identified application.

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Respectfully submitted,

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BRIEF ON APPEAL

Appeal from Group 1645

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I. REAL PARTY IN INTEREST

The real party in interest for this appeal and the present application is Protis Valor Mediterranee, by way of an Assignment recorded in the U.S. Patent and Trademark Office at Reel 014789, Frame 0282.

II. RELATED APPEALS AND INTERFERENCES

There are no prior or pending appeals, interferences or judicial proceedings, known to Appellant, Appellant's representative, or the Assignee, that may be related to, or that will directly affect or be directly affected by or have a bearing upon, the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 1, 10, 11, 15, 25, 29-32, 35-42, 63 and 66-69 are on appeal.

Claims 1, 10, 11, 15, 25, 29-32 and 35-69 are pending.

Claims 43-45 are objected to only for being dependent from a rejected base claim, but are otherwise allowable.

Claims 1, 10, 11, 15, 25, 29-32, 35-42, 63 and 66-69 are rejected.

Claims 46-62, 64 and 65 are withdrawn from consideration.

Claims 2-9, 12-14, 16-24, 26-28, 33 and 34 are canceled.

IV. STATUS OF AMENDMENTS

No Amendment After Final Rejection has been filed. There are no objections to entry of any amendments.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Before the present invention, it has been impossible to isolate and cultivate the known bacterium responsible for Whipple's disease (*Tropheryma Whippelii*) in a manner suitable for performing serological tests. For example, Relman at page 752, first sentence, stated that "Cultivation of the bacillus associated with Whipple's disease, *Tropheryma whippelii*, has been an elusive goal for many generations of clinicians and microbiologists familiar with this disease." Maiwald at page 801, second column, confirmed that "the cultivation of this bacterium has been a goal of clinicians for several decades." Even the Drancourt reference applied in the Office Action refers to "90 years of isolation attempts" at page 9 of the translation. The Bentley and Maiwald references confirm that the present invention solved this longfelt need. See, e.g., Maiwald abstract and Bentley, page 637, col. 2. The applied references do not solve this problem, as confirmed in the subsequent literature, including a subsequent publication by authors of the cited Schoedon reference. See Hinrikson, pp. 1701, 1705 and 1706.

Contrary to all expectations, the present inventors have developed methods of culturing the *Tropheryma Whippelii* bacterium, and thus cultures containing that bacterium in a culture medium. (Page 2, lines 9-13 of the specification.) It is widely recognized that Raoult et al. (i.e., the inventors of the present application) have been the first to teach the suitable conditions to establish the *Tropheryma whipplei* bacterium in culture as acknowledged in Maiwald (see the abstract: "*..many attempts have been made to cultivate this bacterium in vitro. It was eventually isolated, in 2000....*") and Bentley (see page 637 column 2: "*Isolation of the bacterium Tropheryma Whipplei was achieved in 2000, in a long term culture system with human fibroblasts, with a reported generation time of 18 days*" (Raoult et al 2000)). See paragraph 13 of the Drancourt Declaration.

The invention of the present claims is related to the inventors' determination that the *Tropheryma Whippelii* bacterium has an unusually long doubling time, and thus requires a culture medium with both a long lifetime and a long doubling time (page 2, lines 14-18 of the specification). The claimed invention is directed to cultures (claims 1, 30-32, 35-39, 41, 42, 63 and 66-69), isolated antigens (claim 10) and diagnosis methods (claims 11, 15, 25 and 29) made possible by that development. Claims directed to the culturing methods have been withdrawn from consideration.

Claim 1 is directed to a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture (page 3, lines 3-5 of the specification) such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days (page 2, lines 14-17, page 2, line 28 to page 3, line 7, and Examples 1-3 at pages 15-18 of the specification) as detected by inverted microscopy (page 16, lines 16-19 of the specification), wherein the bacterium is *Tropheryma whippelii* (page 2, lines 14-15 of the specification).

Claim 10 is directed to an antigen isolated from the *Tropheryma whippelii* bacterium in the culture according to claim 1 (see page 3, lines 6-7 of the specification), wherein said antigen is a protein of 200 kD determined by polyacrylamide gel electrophoresis using the Western blotting technique (see page 3, lines 10-14 and 25-27 of the specification), which reacts with a specific monoclonal antibody directed against the bacterium *Tropheryma whippelii* responsible for Whipple's disease or an antigen of said bacterium (see page 3, lines 21-27 of the specification), said antibody being produced by a hybridoma deposited in the CNCM of the Institut Pasteur under the Deposit No. I-2411 (see page 3, lines 7-9 and 25-27 of the specification).

Claim 11 is directed to a method for the *in vitro* diagnosis of diseases associated with infections caused by *Tropheryma whippelii* (see page 4, lines 1-3 of the specification),

comprising contacting serum or any other biological fluid of a patient with a culture according to claim 1 or a *Tropheryma whippelii* bacterium obtained from said culture (see page 4, lines 3-6 of the specification), and detecting an immunological reaction (see page 4, lines 7-16 of the specification). See also page 4, line 17 to page 5, line 8.

Claim 63 is directed to a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture (page 3, lines 3-5 of the specification) such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days through successive subcultures (see page 2, lines 14-27, page 2, line 28 to page 3, line 7, and Examples 1-3 at pages 15-18 of the specification), as detected by inverted microscopy (page 16, lines 17-19 of the specification), wherein the bacterium is of the *Tropheryma whippelii* bacterium strain deposited in the CNCM of the Institut Pasteur under Deposit No. I-2202 (see page 3, lines 7-9 of the specification), wherein the bacterium comprises a rpoB gene comprising a partial sequence amplifiable by primers of a sequence identical to SEQ ID NO:4 or 5 (see page 11, lines 26-30 and page 13, line 24 to page 14, line 22 of the specification).

Claim 68 is directed to a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture (page 3, lines 3-5 of the specification) in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and said cell has a dividing time greater than the doubling time of the bacterium (see page 2, line 14 to page 3, line 5 of the specification).

Claim 69 is directed to a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture (page 3, lines 3-5 of the specification) in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and the cell is selected such that it does not multiply so rapidly

relative to the growth of the bacterium as to cause a dilution effect of the bacterium (see page 2, line 14 to page 3, line 5 of the specification).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following grounds of rejection are presented for review:

1) Claims 1, 10, 11, 15, 25, 29-32, 35-39, 41-42, 63 and 66-69 are rejected as not being supported by an adequate written description in compliance with 35 U.S.C. §112, first paragraph;

2) Claims 1, 10, 11, 15, 25, 29-32, 35-39, 41-42, 63 and 66-69 are rejected as non-enabled under 35 U.S.C. §112, first paragraph;

3) Claims 1, 68 and 69 are rejected as anticipated under 35 U.S.C. §102(b) by Schoedon et al., "Deactivation of Macrophages with Interleukin-4 is the Key to the Isolation of *Tropheryma whippelii*," The Journal of Infectious Diseases, Col. 176, pp. 672-677 (1997);

4) Claims 1, 30, 31, 68 and 69 are rejected as anticipated under 35 U.S.C. §102(b) by Muller et al., "Cultivation of T. Whippelii From Peripheral Blood Mononuclear Cells," Gastroenterology, Vol. 116, No. 4, Part 2, Abstract 910 (April 1999);

5) Claims 1, 30, 31, 68 and 69 are rejected as anticipated under 35 U.S.C. §102(b) by Drancourt, La Presse Medicale, Vol. 28: No. 8, pp. 435-39 (Feb. 27, 1999); and

6) Claims 1, 11, 15, 25, 29-32, 35-42, 63 and 66-69 are rejected as having been obvious under 35 U.S.C. §103(a) over Muller, Schoedon or Drancourt in view of Kent, Arch.Pathol.Lab.Med 104 (10), pp. 544-47 (1980) and Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory 1988, Chapters 14/5/6.

VII. ARGUMENT

A. Rejections Under 35 U.S.C. §112, First Paragraph

1. Claims 1, 10, 11, 15, 25, 29-32, 35-39, 41-42, 63 and 66-69 Comply with the Written Description Requirement of 35 U.S.C. §112, First Paragraph

While inaccurately characterizing the current scope of the claims by merely repeating the prior rejection, the Final Rejection does maintain the underlying basis for rejecting the above-listed claims for lack of written description under 35 U.S.C. §112. That basis is essentially that only one strain of *Tropheryma whippelii* has been described in the specification as having been cultured and deposited, and that given that different strains exist, claims encompassing other un-described strains are not supported by the written description.

a. Claim 63

At page 5, lines 14-18 of the Final Rejection, the Examiner points out that claims 43-45, which are limited by reference to the deposited strain, are not included in the subject rejection. Claim 63 is similarly limited by reference to the deposited strain, and thus none of the bases for the §112, first paragraph rejections apply to claim 63. For at least this reason, the rejection of claim 63 is clearly in error and should be reversed.

b. Claims 1, 63, 68 and 69

Claims 1, 63, 68 and 69 are directed to a culture comprising a culture medium and a bacterium responsible for Whipple's disease. The recited bacterium is a species of bacteria known as *Tropheryma whippelii* (also known as *T. Whippelii, T. Whippeli* and *T. Whipplei*). As recited in the subject claims, this bacterium is isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium. The bacterium reproducibly and detectably multiplies in the culture medium for at least 72 days, according to claims 1 and 63. The bacterium is isolated and established in culture in a cell in the culture medium and the cell has a dividing time greater than the doubling time of the

bacterium, according to claim 68. The bacterium is isolated and established in culture in a cell in the culture medium and the cell is selected such that it does not multiply so rapidly relative to the growth of the bacterium as to cause a dilution effect of the bacterium, according to claim 69. Appellants submit that the written description provides ample support for such a culture, which had never been obtained prior to the present invention.

The Final Rejection takes the position that because only a single strain of *Tropheryma whippelii* has been deposited and addressed in Appellants' examples, Appellants' claims must be restricted to that strain. The Final Rejection only justifies this position by exhibiting confusion as to whether it is addressing a species or a strain, and by reference to inapposite case law.

For example, the Final Rejection mischaracterizes Appellants' prior submissions as arguing "that different species of T. Whippelii are known ..." (page 4, third-to-last line) and "applicant correctly pointed that [sic] various species are known in the art" (page 5, line 2). This is not correct. Applicant has consistently stated, as further discussed below, that *T. Whippelii* is a single species, although various strains of it may exist. The Final Rejection further states (page 5, lines 3-4) that "the issue here is whether the specification provided support for all the cultures comprising diverse species of *T. whippelii* or not" and (page 5, lines 6-7) that "claim 1 and the dependent claims ... are not supported by the current specification for the culture comprising diverse species of *T. whippelii.*" Because *T. whippelii* is the name of one species, the reference to "diverse species of *T. whippelii*" clearly reflects confusion over the facts and law relevant to the claims on appeal and the resulting error in the §112 rejections.

Tropheryma whippelii (*T. whippelii*) is a species of bacterium that was known in the art as of the effective filing date of the present application. It has been known that a bacterium is involved with Whipple's disease since 1907. See, e.g., Maiwald, et al.,

"Cultivation of *Tropheryma whippelii* from Cerebrospinal Fluid," Journal of Infectious Disease, vol. 188, pp. 801-808 (September 15, 2003). As described in the specification, the species was observed by electron microscopy as early as 1961, and its phylogenic taxonomy was specified by 1992. Indeed, the bacterial species associated with Whipple's disease was sequenced as early as 1991. See page 1, line 16 to page 2, line 8 of the specification.

The Patent Office incorrectly relies on the holdings in University of California v. Eli Lilly and Co., 119 F.3d 1559 (Fed. Cir. 1997) and Enzo Biochem, Inc. v. Gen-Probe Inc., 323 F.3d 956 (Fed. Cir. 2002) in support of the Final Rejection. However, both Eli Lilly and Enzo are directed to discoveries of genetic materials and uses thereof. Eli Lilly explained that a description of a broad genus of a genetic material, such as cDNA, may be achieved by means such as a recitation of structural features common to all members of the genus. 119 F.3d at 1569. In Enzo, the court held that reference in a patent specification to a deposit of genetic material may be sufficient to describe that genetic material. 323 F.3d at 970.

In contrast, claims 1, 63, 68 and 69 are not directed to a new genetic material, but are instead directed to a culture comprising a culture medium and a known bacterium species, identified by its art-accepted taxonomic name, that is known to be responsible for Whipple's disease. Claims 1, 63, 68 and 69 are not directed to a newly discovered genetic material, the subject of both Eli Lilly and Enzo, but are directed to the selection and combination of a culture medium having certain properties when combined with a known species of bacterium, which allows reproducible and detectable multiplication of that bacterium over time. Thus, Appellants submit that the holdings of Eli Lilly and Enzo do not support the rejection of claims 1, 63, 68 and 69, because claims 1, 63, 68 and 69 are directed to a culture involving a known bacterium, and not a newly discovered genetic material as was the case in Eli Lilly and Enzo.

Contrary to the Patent Office's assertions, it is not necessary to provide more detail regarding the recited bacterium or to limit claims 1, 63, 68 and 69 to a specific deposited strain (although as noted above, claim 63 is so-limited), because *Tropheryma whippelii* was known and well-characterized in the art as of the effective filing date of the present application. The specification only needs to describe in detail that which is new or not conventional. See MPEP §2163(II)(A)(3)(a). As discussed above, it has been known that a bacterium is involved with Whipple's disease since 1907. See also, e.g., Maiwald, et al., "Cultivation of *Tropheryma whippelii* from Cerebrospinal Fluid," Journal of Infectious Disease, vol. 188, pp. 801-808 (September 15, 2003). As described in the specification, the species was observed by electron microscopy as early as 1961, and its phylogenic taxonomy was specified by 1992. Indeed, the *T. whippelii* bacterial species associated with Whipple's disease has been sequenced as early as 1991. See page 1, line 16 to page 2, line 8 of the specification. Thus, those of ordinary skill in the art know what is meant by *Tropheryma whippelii* and require no further written description of it. See, e.g., Enzo, 323 F.3d at 965 (explaining that a biological deposit is not required to meet a written description requirement if the biological material is known).

Indeed, the very law quoted in the Final Rejection establishes the impropriety of the Final Rejection. As stated at page 3, lines 34-39 of the Final Rejection,

> The Enzo court adopted the standard that "the written description requirement can be met by 'show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics .... i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." Id. At 1324, 63 USPQ2d at 1613 (emphasis omitted, bracketed material in original).

Here, the species *Tropheryma whippelii* has been structurally and functionally characterized and sequenced in the prior art, as described in detail at page 1, line 16 through page 2, line 11 of the specification. There is no suggestion, much less evidence or record, that its various

strains do not at least share significant partial structure, physical properties, chemical properties and functional characteristics, or that they do not include the known correlation between the known structure and function of the species. To the contrary, this is essentially definitional as to strains of a species, and the subject claims explicitly specify that the recited "bacterium responsible for Whipple's disease" is the species "*Tropheryma whippelii*." Thus the disclosure of the species by name, coupled with the detailed characterization of that species in the prior art as described in the present specification, is more than adequate written description to support the full scope of the subject claims.

Nor would persons of ordinary skill in the art have needed to read about any additional strains of *Tropheryma whippelii* or have required the deposit of any additional strains of *Tropheryma whippelii* in order to understand that the claimed invention is fully described by the present specification. As discussed above, claims 1, 63, 68 and 69 focus on the combination of a culture medium having certain properties with the known *Tropheryma whippelii* bacterium. There is no suggestion, much less citation of evidence or reasonable scientific rationale, in the record that differences among strains of *Tropheryma whippelii* would have any effect on the practice of the invention of claims 1, 63, 68 and 69 or on the understanding of those of ordinary skill in the art that the invention of claims 1, 63, 68 and 69 is fully described in the specification. The Office Action's statement that different strains would have some differences in their sequences and proteins provides no suggestion that such differences would have been expected to have any effect on the practice of the invention of claims 1, 63, 68 and 69.

The non-prior art Maiwald reference suggests that its authors observed a doubling time of 4 days for *Tropheryma whippelii*, as opposed to the 18-day initial doubling time observed by Appellants, but still characterizes that time as "among the longest observed doubling time for any bacteria." Maiwald characterizes the difference in calculated doubling

times as being "due either to the different measurement methods or culture conditions or to the difference between *Tropheryma whippeli* strains." In any case, in view of Appellants' first disclosure of the unusually long doubling times of *Tropheryma whippelii* and the fact that they are longer than those of the monocytes used in the unsuccessful prior art attempts to culture *T. whippelii* as discussed below, one of ordinary skill in the art would have readily understood that the teachings of the invention are applicable to all strains of *T. whippelii*.

For all of the above reasons, reversal of the written description rejection of claims 1, 63, 68 and 69 is respectfully requested.

c. Claims 11, 15, 30-32, 35-39, 41-42, 63 and 66-69

Claims 11, 15, 30-32, 35-39, 41-42, 63 and 66-69 depend from claim 1. For the same reasons as discussed above regarding claim 1, the reference to *Tropheryma whippelii* without further limitation to a deposited strain does not render them subject to rejection for lack of written description under 35 U.S.C. s112, first paragraph. No additional basis for rejecting these claims under §112 has been presented in the Final Rejection. Thus reversal of the written description rejection of these claims is also respectfully requested.

d. Claims 10, 25 and 29

Antigen claim 10 and its dependent method claims 25 and 29 also are supported by an adequate written description in compliance with the written description requirement of 35 U.S.C. §112.

Claim 10 recites an antigen isolated from the *Tropheryma whippelii* bacterium in the culture according to claim 1. The specification clearly describes that the bacterium isolated and established in culture as recited in claim 1 may be utilized as an antigen source. See page 3, lines 6-7 of the specification. It was also well known in the art that isolated and cultured bacteria may be used as antigen sources, and it was known how to isolate an antigen once such a culture was obtained. Furthermore, an example of obtaining such an antigen is fully

described in Example 6 of the specification. See pages 19-23 of the specification. The Final Rejection presents no evidence or scientific rationale for assuming that differences among strains would alter these fundamental properties of the *Tropheryma whippelii* bacterium.[1]

Furthermore, claim 10 itself specifies the size and reactivity of the antigen and the source of the deposited antibody with which it is reactive, as well as the fact that it is isolated from the *Tropheryma whippelii* bacterium in the culture according to claim 1. Thus no further written description is required to support these claims, and reversal of the rejection of these claims is respectfully requested.

e. Claims 11 and 15

Claim 11 and its dependent claim 15 recite a method for the in vitro diagnosis of diseases associated with infection caused by *Tropheryma whippelii*, comprising contacting serum or any other biological fluid of a patient with a culture according to claim 1 or a *Tropheryma whippelii* bacterium obtained from said culture, and detecting an immunological reaction. For the same reasons as discussed above with respect to claim, these claims also are supported by an adequate written description in compliance with the written description requirement.

In addition, Example 6 of the specification clearly describes how methods as recited in claims 11 and 15 were utilized for in vitro diagnosis of 15 patients. Further, the specification, beginning on page 4, clearly describes methods for the in vitro serological diagnosis of infections caused by *Tropheryma whippelii*, wherein the bacterium, an antigen of the bacterium or a specific antibody is brought into contact with a sample taken from the patient and consisting of a human serum, biological fluid or swab. Appellants thus submit that claims 11 and 25 also are supported by an adequate written description in compliance with

[1] Again, the Final Rejection exhibits confusion as to what is claimed, arguing that "each antigen obtained from diverse species of *T. whippelii* is different and distinct" (page 5,

the written description requirement, and reversal of the rejection of these claims is also respectfully requested.

f. Conclusion

Claims 1, 10, 11, 15, 25, 29-32, 35-39, 41-42, 63 and 66-69 are supported by an adequate written description in compliance with the written description requirement of §112, first paragraph. Accordingly, the written description rejections of these claims should be reversed.

2. Claims 1, 10, 11, 15, 25, 29-32, 35-39, 41-42, 63 and 66-69 Comply with the Enablement Requirement of 35 U.S.C. §112, First Paragraph

a. Claims 1, 10, 11, 15, 25, 29-32, 35-39, 41-42, 63 and 66-69

The entire basis for the enablement rejection is the single sentence at page 5, lines 16-18 of the Final Rejection "Since the claims do not provide support for the broadly claimed culture, the claims stand rejected under enablement rejection [sic] for the reasons set forth in the written description rejection as discussed above." The Patent Office provides no additional support or explanation of the enablement rejection. This is an improper application of the law. It is well settled that the written description requirement is separate and distinct from the enablement requirement. See MPEP §2161. Thus, the Patent Office's brief statement that the claims are rejected under the enablement requirement for the same reasons as the rejection of the claims under the written description requirement is improper. First, the stated rationale is not a basis for an enablement rejection. The issue is whether one of ordinary skill in the art would have been able to practice the invention as claimed. There has been no allegation, much less presentation of evidence or scientific rationale, in the Office Action that such a person would not have been able to practice the invention as

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last paragraph). As noted above, *T. whippelii* is a single species.

claimed with any strain of *Tropheryma whippelii*. For this reason alone, the rejection should be withdrawn.

b. Claim 63

As noted above, there is no basis at all stated in the Office Action for the written description rejection of claim 63. Thus the enablement rejection that relies on the written description rejection of this claim must also be reversed.

c. Claims 1, 11, 15, 30-32, 35-39, 41-42, 63 and 66-69

The present specification clearly discloses a culture comprising a culture medium and a *Tropheryma whippelii* bacterium responsible for Whipple's disease as required in claims 1, 63, 68 and 69 and the claims depending from claim 1. One of ordinary skill in the art would have been able to utilize the teachings of the present specification, including the examples, to create such a culture comprising a culture medium and a bacterium responsible for Whipple's disease.

For example, the specification explains that a cell culture in which *Tropheryma whippelii* is to be isolated and multiplied, must have both a long lifetime and a slow multiplication time, because the doubling time of the bacterium is very long, such as 18 days. See page 2, lines 14-18 of the specification. Further, if the cells of such a culture multiply too rapidly relative to the growth of the bacterium, the bacterium cannot be cultivated because a dilution effect takes place and it becomes impossible to segregate the infected cells from the non-infected cells. See page 2, lines 25-27 of the specification. Thus the specification provides sufficient detail that one of ordinary skill in the art would have been enabled to make and use the culture recited in claims 1, 63, 68 and 69 and the claims depending from claim 1 given the information that is provided in the present specification. In fact, the record shows that those of ordinary skill in the art have actually been enabled by the Raoult disclosure (the first named inventor of the present application) to practice the invention. See,

e.g., Bentley, et al., "Sequencing and Analysis of the Genome of the Whipple's Disease Bacterium *Tropheryma whipplei*," The Lancet, Vol. 361, pp. 637644 (February 22, 2003).

For all of the above reasons, the enablement rejection of these claims should be reversed.

d. Claims 10, 25 and 29

With respect to the antigen-related claims, one of ordinary skill in the art would have understood that every bacterium that is isolated and established in culture is also an antigen source. The present disclosure also describes use of the bacterium isolated and established in the culture of the present claims as an antigen source, and identifies as an exemplary antigen the protein of claim 10 having a molecular weight of about 200 kD determined by polyacrylamide gel electrophoresis. See, for example, page 3, lines 10-14 of the specification. Such a person would also have clearly been able to test it for reactivity with the specified antibody from the identified deposited hybridoma. The Final Rejection has stated no basis, much less provided evidence or a scientific rationale, as to why one of ordinary skill in the art would not have been able to practice this invention with antigens of any strain of *Tropheryma whippelii* bacterium from the culture of claim 1. Thus the enablement rejection of these claims should also be reversed.

e. Claims 11, 15, 25 and 29

The specification further describes exemplary methods for the in vitro serological diagnosis of infection caused by *Tropheryma whippelii*, wherein the bacterium, an antigen of the bacterium or a specific antibody is brought into contact with a sample taken from the patient and consisting of human serum, biological fluid or a swab. Such methods are described throughout the specification, for example at pages 4-8 of the specification.

Moreover, the Examples set forth in the specification are sufficiently detailed to ensure that one of ordinary skill in the art would have been enabled to practice the claimed invention.

Thus for this reason also, the enablement rejection of these claims should be reversed.

f. Conclusion

For at least the foregoing reasons, Appellants submit that claims 1, 10, 11, 15, 25, 29-32, 35-39, 41-42, 63 and 66-69 are enabled by the specification, and the enablement rejection under 35 U.S.C. §112, first paragraph should be reversed.

B. Rejections Under 35 U.S.C. §102(b)

1. Claims 1, 68 and 69 are Not Anticipated by Schoedon

Claims 1, 68 and 69 are rejected as anticipated under 35 U.S.C. §102(b) by Schoedon et al., "Deactivation of Macrophages with Interleukin-4 is the Key to the Isolation of *Tropheryma whippelii*," The Journal of Infectious Diseases, 176:672-77 (1997) ("Schoedon").

The Patent Office alleges that Schoedon teaches or suggests all of the features recited in claims 1, 68 and 69. Appellants respectfully disagree.

Schoedon teaches a culture comprising a cell medium of monoblasts from the cell line SigM5, which is derived from bone marrow. See pages 673 and 674 of Schoedon. As is known to one of ordinary skill in the art, immortalized cell lines derived from monoblasts and monocytes have a very short doubling time. For example, mononuclear bone marrow cells divide approximately six times during a culturing period of 14 days. See page 36 of Helin, et al., "Measles Virus Replication in Cells of Myelomonocytic Lineage is Dependent on Cellular Differentiation Stage," Virology, vol. 253, pp. 35-42 (1999). Further, it is known that cells from human promyelocytic leukemia cell line HL60 have a doubling time of 34.0 hours. See the Abstract of Foa, et al., "Growth Pattern of the Human Promyelocytic Leukemia Cell Line HL60," Cell Tissue Kinet. Vol. 15, no. 4, pp. 399-404 (July 1982).

As evidenced in the Drancourt Declaration, there is doubt whether Schoedon actually cultured the *Tropheryma whippelii* bacterium recited in claims 1, 68 and 69.

First, in paragraph 7 of the Drancourt Declaration, it is explained that with the requirements for the identification of bacteria by molecular biology, the results published by Schoedon would not be accepted as definite evidence that they have successfully cultivated the *Tropheryma whipplei* bacterium. Indeed, in their article the identification of the bacterium is based on a PCR with primers drawn from the 16S r DNA of the *Tropheryma whipplei* bacterium, which are universal primers and not considered as sufficiently specific of any bacterium. As acknowledged by Hinrikson, Dutly and Altwegg in their article of 1999 (Hinrikson, page 1701 column 2): "Several diagnostic *T. whippelii*-PCRs that target parts of the 16S rDNA have been established (Relman *et al.* 1992; . . Altwegg *et al.* 1996. . .). However, such systems may not discriminate between closely related species...". In addition, the PCR products obtained by Schoedon have not been sequenced, as now required for reliable identification of a new bacterium, but they have only been subjected to Southern blotting using an oligonucleotide of the 16S rRNA gene. The primers used by Schoedon were published by Relman (reference 4 of Schoedon), Maiwald (reference 13 of Schoedon) and Altwegg (reference 12 of Schoedon) and these authors have stated that they could not reproduce the experiments of Schoedon (see Maiwald and Hinrikson).

Second, paragraph 8 of the Drancourt Declaration goes on to explain that the kinetics results reported by Schoedon are not compatible with the more recent data concerning this bacterium. Indeed, reported kinetics of growth such as in Figure 4 and Figure 5 of Schoedon are absolutely not compatible with what was later known about the growth kinetics of *Tropheryma whippelii*. In the histogram of Figure 4 as well as in the photographs of Figure 5, they report passing from about 15-20% of cells infected by the bacteria to more than 50% in 48 hr. Such kinetics implies a doubling time of the bacteria lower than 24 hr. Such result is

an extraordinarily rapid growth while *Tropheryma whippelii* is on the contrary particularly slow in growth. At the time, it was not known that the doubling time of the *Tropheryma whippelii* bacterium was significantly longer than that of the cells utilized in Schoedon.

In fact, Relman even theorized that Schoedon may have propagated a close relative of *Tropheryma whippelii* bacterium. See Relman at page 753, second paragraph. Thus, it is likely that an organism mimicking some of the *Tropheryma whippelii* characteristics (intracellular growth, bacillary morphology, periodic acid-schiff positivity, reactivity with "TW-1/TW-3 or TW-1/TW-2" PCR primers) contaminated the Schoedon culture leading to Schoedon's conclusion that *Tropheryma whipplei* had been isolated and cultured. See paragraph 9 of the Drancourt Declaration.

a. Claim 1

Schoedon does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days, as required in claim 1.

Due to the short doubling time of the cells in the culture medium of Schoedon, these cells would double prior to the *Tropheryma whippelii* bacterium doubling even one time. Thus, the culture taught by Schoedon does not comprise a culture medium and bacterium that can reproducibly and detectably multiply in the culture medium for at least 72 days, as required in claim 1. To the contrary, the product of Schoedon would be diluted well below detectable limits in far less time. One of ordinary skill in the art would have understood that the term "detectably" in the context of claim 1 and the present application reflects that the bacteria do not become increasingly diluted over time in the culture medium. In contrast, as demonstrated below, when the doubling time of cells in the culture medium is too short as in

the applied references, the bacteria grown in such cells become dilute and undetectable over time.

This deficiency of Schoedon is graphically demonstrated in the Table below. This Table demonstrates the dilution effect when *Tropheryma whippelii* is cultured in a medium having a doubling time of two days (48 hours). A doubling time of two days exemplifies the outcome of culturing *Tropheryma whippelii* in a culture medium having a doubling time significantly shorter than the initial doubling time (18 days) of the bacterium, yet still longer than the doubling time of the cells used in the Schoedon medium.

Day	Number of *Tropheryma whippelii* bacteria per number of cells in culture, where the bacteria have a doubling time of 18 days and the cells have a doubling time of 2 days
0	2 bacteria per 2 cells
2	2 bacteria per 4 cells
4	2 bacteria per 8 cells
6	2 bacteria per 16 cells
8	2 bacteria per 32 cells
10	2 bacteria per 64 cells
12	2 bacteria per 128 cells
14	2 bacteria per 256 cells
16	2 bacteria per 512 cells
18	2 bacteria per 512 cells
20	2 bacteria per 1024 cells

Extrapolating out to 72 days, it is readily apparent that a culture even in short-doubling-time cells with longer doubling times than those of Schoedon does not meet the requirements of claim 1. The increasing dilution of the bacteria would prevent the bacterium

from detectably multiplying over time in the culture medium for at least 72 days, in contrast to the requirements of claim 1.

Further, Schoedon teaches that the culture medium includes monocyte cells, which have a lifetime of 30 days. The present specification explains that this lifetime is insufficient in view of the doubling time of the bacterium. See page 2, lines 19-24 of the specification. Such a short lifetime of the Schoedon cell medium in combination with the short doubling time of the cells in the Schoedon medium, in comparison to the doubling time of the bacterium, indicates that the amount of bacteria in the cell medium of Schoedon is not sufficient to create a culture having a culture medium and a bacterium that can reproducibly and detectably multiply over time in the culture medium for at least 72 days.

This fact is emphasized in the subsequent literature, including publications by Schoedon's own research group. Other teams involved in this technical field have also unsuccessfully attempted to confirm the results of Schoedon without success. In particular, as described in Maiwald, the finding by Schoedon of the propagation of bacteria could not be confirmed in subsequent studies. Maiwald, p. 802, col. 1, lines 4-9: "*...investigators inoculated interleukin-4-deactivated macrophages with heart-valve tissue affected by WD and reported propagations [8].*[2] *However, this finding could not be confirmed in subsequent studies [9].*"[3] See paragraph 4 of the Drancourt Declaration.

Two of the authors of Schoedon, Martin Altwegg and Fabrizio Dutly, indicated in an article published about two years after Schoedon (Hinrikson) that the relationship between clinical manifestations of Whipple's disease and different infecting strains of *Tropheryma whippeli* has not been studied "*mainly because of the absence of reliable cultures* (Schoedon *et al.*, 1997)." Hinrikson, p. 1705, col. 1, discussion. In fact, the summary of this article on

[2] Reference [8] of Maiwald is the Schoedon reference.

page 1701 refers to *Tropheryma whippelii* as "*the uncultivated causative agent of Whipple's disease*" (emphasis added). In addition, the article indicates on page 1706 that certain studies are "*not feasible due to the fact that 'T. whippelii' has not yet been cultured on artificial media.*" See paragraph 4 of the Drancourt Declaration.

See also the attached Maiwald article where the Zaaijer reference is characterized as disclosing that "*Tropheryma whippelii* is easily ingested by interleukin-4-deactivated macrophages, but does not multiply."

As further explained in paragraph 4 of the Drancourt Declaration, the failure of Schoedon to culture the *Tropheryma whippelii* bacterium was confirmed by two of the authors of Schoedon, Martin Altwegg and Fabrizio Dutly, who indicated in an article published about two years after Schoedon (Hinrikson), that the relationship between clinical manifestations of Whipple's disease and different infecting strains of *Tropheryma whippeli* has not been studied "*mainly because of the absence of reliable cultures* (Schoedon *et al.*, 1997)." Hinrikson, p. 1705, col. 1, discussion. In fact, the summary of this article on page 1701 refers to *Tropheryma whippelii* as "*the uncultivated causative agent of Whipple's disease*" (emphasis added). In addition, the article indicates on page 1706 that certain studies are "*not feasible due to the fact that 'T. whippelii' has not yet been cultured on artificial media*". See also Raoult et al., The New England Journal of Medicine, Vol. 342, No. 9, pp. 620-625 (March 2, 2000), which indicates that the isolate described in Schoedon "*could not be subcultured*" (p. 620, col. 2). No one has been able to reproduce the results reported in Schoedon. See, for example, paragraphs 3 and 12 of the Drancourt Declaration, where Professor Drancourt explains that he was unable to reproduce the results of Schoedon and that

[3] Reference [9] of Maiwald is the Zaaijer reference.

his article merely summarized the work of researchers in the field such as reported in the scientific literature, including Schoedon.

During the December 8, 2006 interview, the Examiner suggested that Schoedon teaches that the cultures of *Tropheryma whippelii* in the culture medium are passaged every 8 to 10 days (see page 673, second column of Schoedon), and that allegedly there would be more *Tropheryma whippelii* bacterium present in the culture with each passage. Appellants respectfully disagree with this allegation. Specifically, with each passage of the culture medium taught by Schoedon, the cells of the culture medium would multiply while the number of bacteria present would remain constant. As such, over time, the bacterium in the culture would rapidly become undetectable because it would have been diluted by the too frequent passaging, especially coupled with the too-short doubling time of the cells in which the bacteria are grown.

Moreover, as explained in the Declaration Under 37 CFR §1.132 submitted September 9, 2005, Schoedon does not report the presence of *Tropheryma whippelii* after more than 4 passages. See paragraph 10 of the Drancourt Declaration. Thus, Schoedon does not teach or suggest a bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days, as required in claim 1. To the contrary, Schoedon at best teaches that the bacteria rapidly become undetectable in the Schoedon medium, as discussed above. See, for example, paragraph 10 of the Drancourt Declaration, where Professor Drancourt explains that one of ordinary skill in the art knows that to establish an intracellular bacterium in culture by using ex-vivo cells as did Schoedon, one can not obtain multiplication of the bacteria if the incubation period between two passages is lower than the doubling time of the bacterium. Under such conditions, one of ordinary skill would obtain a dilution of the bacterium as the

ratio bacteria/cells necessarily decreases and you will not be able to detect any bacterium after 3 or 4 passages, depending on the amount of bacteria in the initial inoculum.

Thus the rejection of claim 1 under 35 U.S.C. §102(b) over Schoedon should be reversed.

b. Claim 68

Schoedon does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and said cell has a dividing time greater than the doubling time of the bacterium, as required in claim 68. As discussed above, Schoedon teaches a culture comprising a cell medium of monoblasts, which have a very short doubling time of under two days. The bacterium *Tropheryma whippelii*, on the other hand, has one of the longest doubling times of all bacteria, well over two days. See, e.g., Maiwald, pages 802 and 806. Thus Schoedon does not satisfy the limitations of claim 68, and the §102(b) rejection of that claim over Schoedon should also be reversed.

c. Claim 69

Schoedon does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and the cell is selected such that it does not multiply so rapidly relative to the growth of the bacterium as to cause a dilution effect of the bacterium, as required in claim 69. As discussed above, the monoblast cells of Schoedon multiply more rapidly than the *Tropheryma whippelii* bacterium of claim 69. As graphically demonstrated in the table above, and as is true for a cell having a doubling time that is at all greater than the doubling time of the bacterium, when the cell has a shorter doubling time than the doubling time of the bacterium, the bacterium is

necessarily diluted in the culture. Thus Schoedon does not satisfy the limitations of claim 69, and the §102(b) rejection of that claim over Schoedon should also be reversed.

d. Claims 1, 68 and 69

Furthermore, Schoedon is not an enabling reference, and is thus improperly applied as an anticipatory reference under 35 U.S.C. §102(b). A patent claim cannot be anticipated by a prior art reference if the allegedly anticipatory disclosures are not enabling to one of ordinary skill in the art. See, for example, Elan Pharm., Inc. v. Mayo Found. for Med. Educ. & Research, 346 F.3d 1051, 1054 (Fed. Cir. 2003). As evidenced by the Drancourt Declaration Under 37 CFR §1.132 submitted September 9, 2005 and Maiwald, et al., it has not been possible to reproduce or confirm the teachings of Schoedon in subsequent studies. See paragraphs 3, 4 and 12 of the Drancourt Declaration. In fact, Schoedon authors themselves have referred to the absence of reliable cultures of *Tropheryma whippelii*, and have referred to it as "uncultivated" in the Hinrikson article of record as discussed in paragraph 4 of the Drancourt Declaration of record. See, e.g., Hinrikson et al. "Detection of three different types of Tropheryma whippelii" in International Journal of Systematic Bacteriology (1999; 49:1701-1706). In fact, as discussed at length above, it has been posited that Schoedon may not even have been observing *Tropheryma whippelii*. See, e.g., Relman, "Editorial: The Whipple Bacillus Lives (Ex Vivo)," The Journal of Infectious Diseases, Vol. 177, pp. 752-754 (1997). See paragraph 9 of the Drancourt Declaration.

As discussed above, various teams involved in this technical field have unsuccessfully attempted to confirm the results of Schoedon. In particular, as described in Maiwald, the finding by Schoedon of the propagation of bacteria could not be confirmed in subsequent studies. Maiwald, p. 802, col. 1, lines 4-9: "*...investigators inoculated interleukin-4-deactivated macrophages with heart-valve tissue affected by WD and reported propagations*

[Schoedon]. However, this finding could not be confirmed in subsequent studies [Zaaijer]." See paragraph 4 of the Drancourt Declaration

Two of the authors of Schoedon, Martin Altwegg and Fabrizio Dutly, indicated in an article (Hinrikson) published about two years after Schoedon, that the relationship between clinical manifestations of Whipple's disease and different infecting strains of *Tropheryma whippeli* has not been studied "*mainly because of the absence of reliable cultures* (Schoedon *et al.*, 1997)." Hinrikson, p. 1705, col. 1, discussion. In fact, the summary of this article on page 1701 refers to *Tropheryma whippelii* as "*the uncultivated causative agent of Whipple's disease*" (emphasis added). In addition, Hinrikson indicates on page 1706 that certain studies are "*not feasible due to the fact that 'T. whippelii' has not yet been cultured on artificial media.*" See paragraph 4 of the Drancourt Declaration. The Raoult team also tried but failed to reproduce the Schoedon results. See paragraph 3 of the Drancourt Declaration.

Moreover, that a culture according to the teachings of Schoedon could not be produced is evident by the fact that a culture was never deposited in an official deposit collection. As one of ordinary skill in the art is aware, successful cultures of bacteria that have been the subject of research for approximately ninety years would have been deposited in an official deposit collection to demonstrate the successful culture of *Tropheryma whippelii* bacterium. See paragraph 7 of the Drancourt Declaration.

Further, the kinetics results reported by Schoedon are not compatible with the more recent data concerning this bacterium. Indeed, as discussed above, reported kinetics of growth such as in Figure 4 and Figure 5 of Schoedon are absolutely not compatible with what was later known about the growth kinetics of *Tropheryma whipplei*. In the histogram of Figure 4 as well as in the photographs of Figure 5, Schoedon reports passing from about 15-20% of cells infected by the bacteria to more than 50% in 48 hr. Such kinetics implies a doubling time of the bacteria of lower than 24 hr. Such a result is an extraordinarily rapid

growth while *Tropheryma whipplei* is on the contrary particularly slow in growth. At the time, it was not known that the doubling time of the *Tropheryma whipplei* bacterium was unusually long. See paragraph 8 of the Drancourt Declaration.

As discussed in paragraph 1 of the Drancourt Declaration, any culture that was obtained according to the teachings of Schoedon was abandoned as recognized by Professor Schoedon in the letter attached to the Declaration. Also, none of the isolates of Schoedon has been made available in a strain deposit collection contrary to international code in microbiology, which asks that new isolates be deposited in public collection especially where it has long been sought to obtain such cultures by scientists in the area.

Further as discussed at length above, and in paragraphs 3, 4 and 12 of the Drancourt Declaration, all attempts to replicate the results of Schoedon have been unsuccessful and a culture of *Tropheryma whippelii* bacterium according to the teachings of Schoedon could not be obtained. Thus Schoedon is clearly non-enabling as to the claimed invention.[4]

Finally, in the Final Rejection, the Patent Office alleges that because "the Patent Office does not have the facilities for examining and comparing applicants' product with the product of the prior art, the burden is on applicant to show a novel or unobvious difference between the claimed product and the product of the prior art" (see page 6 of the Final Rejection). It is clear from this statement that the Patent Office has completely ignored and/or disregarded the numerous evidentiary articles and the Drancourt Declaration provided by the Appellant to demonstrate the differences between the culture of claims 1, 68 and 69 and the culture of Schoedon. Specifically, many of the evidentiary articles and the Drancourt Declaration submitted by the Appellant clearly demonstrate that the teachings of Schoedon are not enabling and that Schoedon does not teach or suggest a culture comprising a culture

[4] In contrast, those skilled in the art have been enabled by the disclosure of the present inventors to practice the claimed invention. See paragraph 6 of the Drancourt Declaration.

medium and a bacterium responsible for Whipple's disease, such that (1) the *Tropheryma whippelii* bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days, as required in claim 1, (2) the cell in the culture has a dividing time greater than the doubling time of the *Tropheryma whippelii* bacterium, as required in claim 68, and (3) the cell in the culture is selected such that it does not multiply so rapidly relative to the growth of the *Tropheryma whippelii* bacterium as to cause a dilution effect of the bacterium, as required in claim 69.

e. Conclusion

For at least the foregoing reasons, Schoedon does not teach or suggest all of the features recited in any of claims 1, 68 and 69. Thus the §102(b) rejection of those claims should be reversed.

2. Claims 1, 30, 31, 68 And 69 are Not Anticipated by Muller

Claims 1, 30, 31, 68 and 69 are rejected as anticipated under 35 U.S.C. §102(b) by Muller et al., "Cultivation of T. Whippelii From Peripheral Blood Mononuclear Cells," Gastroenterology, Vol. 116, No. 4, Part 2, Abstract 910 (1999) ("Muller").

Muller teaches a culture comprising a culture medium including cells of the monocyte cell line U937. As discussed above, monocyte cells have a doubling time that is significantly shorter than the doubling time of *Tropheryma whippelii*. As explained above and as demonstrated by the Table set forth above, such a discrepancy between the doubling time of the cells in the Muller culture medium and the doubling time of the *Tropheryma whippelii* causes the *Tropheryma whippelii* to rapidly become dilute and undetectable in the culture medium over time.

Further, similar to Schoedon discussed above, Muller also does not provide an enabling disclosure for a successful culture of *Tropheryma whippelii*. Muller purports to describe the cultivation of *Tropheryma whippelii* in peripheral blood mononuclear cells

(PMNC) treated with IL-4 and the cocultivation of *Tropheryma whippelii* in PMNC with macrophages and with the monocytic cell line U937, both deactivated by IL-4 pretreatment. The abstract indicates that its results "*were positive suggesting true bacterial growth in those cells*" and that treatment with IL-4 "*seems to induce replication of T. whippelii*" (emphasis added). Based on use of the terms "*suggesting*" and "*seems*," it is clear that the authors could not conclusively say that the bacteria had reproducibly multiplied in this culture. Appellants note that this abstract has never been followed by a corresponding scientific publication or a deposit, which would normally have followed such an important development as the successful culturing of *Tropheryma whippelii*. See paragraphs 1 and 2 of the Drancourt Declaration.

Further, as detailed in paragraphs 3, 4 and 12 of the Drancourt Declaration, similar to Schoedon, attempts to replicate the results of Muller have been unsuccessful and a culture of *Tropheryma whippelii* bacterium according to the teachings of Muller could not be obtained.

(1) Claim 1

Muller fails to teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days as detected by inverted microscopy, wherein the bacterium is *Tropheryma whippelii*, as recited in claim 1

Muller teaches that *Tropheryma whippelii* from peripheral blood mononuclear cells were cultivated without antibiotics with IL-4 for 10 days. In other words, Muller teaches that the bacterium *Tropheryma whippelii* was isolated and detectable for only 10 days. Furthermore, as discussed above with respect to Schoedon, it is not inherent that the Muller bacteria would remain detectable for 72 days. To the contrary, it is clear that they would not

do so. Thus Muller does not satisfy the limitations of claim 1, and the §102(b) rejection of that claim over Muller should also be reversed for that reason.

(2) Claim 68

Muller does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and said cell has a dividing time greater than the doubling time of the bacterium, as required in claim 68. To the contrary, Muller teaches using monocyte cells, which have a doubling time shorter than that of the *Tropheryma whippelii* bacterium. Thus Muller does not satisfy the limitations of claim 68, and the §102(b) rejection of that claim over Muller should also be reversed for that reason.

(3) Claim 69

Muller does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and the cell is selected such that it does not multiply so rapidly relative to the growth of the bacterium as to cause a dilution effect of the bacterium, as required in claim 69. To the contrary, Muller teaches using monocyte cells, which have a doubling time shorter than that of the *Tropheryma whippelii* bacterium, and thus which inherently cause a dilution effect of the bacterium as the cells multiply more rapidly than the bacteria in them. Thus Muller does not satisfy the limitations of claim 69, and the §102(b) rejection of that claim over Muller should also be reversed for that reason.

(4) Claim 30

As explained above, Muller teaches that the medium includes immortalized monocyte cells, i.e., monocyte cell line U937. Thus, Muller also does not teach or suggest that the

culture is not a cell culture in monocyte cells, as recited in claim 30. Thus Muller does not satisfy the limitations of claim 30, and the §102(b) rejection of that claim over Muller should also be reversed for that reason, as well as for its dependency on claim 1.

(5) Claim 31

As explained above, Muller teaches that the medium includes immortalized monocyte cells, i.e., monocyte cell line U937. Thus, Muller also does not teach or suggest that the culture is a cell culture in immortalized cells other than monocyte cells, as recited in claim 31. Thus Muller does not satisfy the limitations of claim 31, and the §102(b) rejection of that claim over Muller should also be reversed for that reason, as well as for its dependency on claim 1.

b. Conclusion

For at least the foregoing reasons, Muller is not prior art under §102(b) and does not teach or suggest all of the features recited in any of claims 1, 30, 31, 68 and 69. Accordingly, the §102(b) rejection of those claims over Muller should be reversed.

3. Claims 1, 30, 31, 68 and 69 are Not Anticipated by Drancourt

Claims 1, 30, 31, 68 and 69 are rejected as anticipated under 35 U.S.C. §102(b) by Drancourt, La Presse Medicale, Vol. 28: No. 8, pp. 435-39 (Feb. 27, 1999) ("Drancourt").

The Patent Office alleges that Drancourt teaches or suggest all of the features recited in claims 1, 30, 31, 68 and 69. Appellants respectfully disagree.

As previously discussed and set forth in paragraph 12 of the Declaration of Professor Drancourt, the author of the Drancourt article, the Drancourt article merely provides a summary of various articles concerning *Tropheryma whippelii*. It does not set forth the results of any additional experimentation. In particular, the only article relating to cultivation of *Tropheryma whippelii* mentioned in Drancourt, Reference No. 13, is the Schoedon

reference discussed above. Thus Drancourt adds nothing to the teachings of Schoedon, discussed above.

As confirmed by Professor Drancourt himself in paragraph 12 of the Declaration of record, the Drancourt reference does not reflect any experimentation that was conducted to confirm the accuracy or repeatability of the work described in Schoedon. Instead, this paper merely summarizes the statements of researchers in the field as reported in the scientific literature, including Schoedon. Even so, the Drancourt reference stated at page 9 of the translation that such work needs to be confirmed before being adopted. As detailed in the Drancourt Declaration, the Schoedon work has not been confirmed in spite of efforts in the art to confirm it, and even the authors of the Schoedon reference were unable to come up with reliable cultures of *Tropheryma whippelii* in their later work. See paragraphs 3 and 4 of the Drancourt Declaration.

The Patent Office again alleges that because "the Patent Office does not have the facilities for examining and comparing applicants' product with the product of the prior art, the burden is on applicant to show a novel or unobvious difference between the claimed product and the product of the prior art" (see pages 7-8 of the Final Rejection). As explained above, Drancourt merely provides a summary of various articles concerning *Tropheryma whippelii*, and including providing a summary of Schoedon. However, as explained in more detail above with respect to Schoedon, the Patent Office has completely ignored and/or disregarded the numerous evidentiary articles and the Drancourt Declaration provided by the Appellant to demonstrate the differences between the culture of claims 1, 30, 31, 68 and 69 and the culture of Schoedon. Appellant has therefore already met its burden of showing a novel or unobvious difference between the claimed product and the product of the prior art.

a. Claim 1

Because Drancourt merely summarizes Schoedon, for all of the reasons discussed above with respect to Schoedon, Drancourt does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days, as required in claim 1. To the contrary, Schoedon, and thus Drancourt, at best teaches that the bacteria rapidly become undetectable in the Schoedon medium, as discussed above. Thus the rejection of claim 1 under 35 U.S.C. §102(b) over Drancourt should be reversed.

b. Claim 68

Because Drancourt merely summarizes Schoedon, for all of the reasons discussed above with respect to Schoedon, Drancourt does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and said cell has a dividing time greater than the doubling time of the bacterium, as required in claim 68. As discussed above, Schoedon, and thus Drancourt, teaches a culture comprising a cell medium of monoblasts, which have a very short doubling time of under two days. The bacterium *Tropheryma whippelii*, on the other hand, has one of the longest doubling times of all bacteria, well over two days. Thus Drancourt does not satisfy the limitations of claim 68, and the §102(b) rejection of that claim over Drancourt should also be reversed.

c. Claim 69

Because Drancourt merely summarizes Schoedon, for all of the reasons discussed above with respect to Schoedon, Drancourt does not teach or suggest a culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being

isolated and established in culture in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and the cell is selected such that it does not multiply so rapidly relative to the growth of the bacterium as to cause a dilution effect of the bacterium, as required in claim 69. As discussed above, the monoblast cells of Schoedon, and thus Drancourt, multiply more rapidly than the *Tropheryma whippelii* bacterium of claim 69. As graphically demonstrated in the table above, and as is true for a cell having a doubling time that is at all greater than the doubling time of the bacterium, when the cell has a shorter doubling time than the doubling time of the bacterium, the bacterium is necessarily diluted in the culture. Thus Drancourt does not satisfy the limitations of claim 69, and the §102(b) rejection of that claim over Drancourt should also be reversed.

d. Claim 30

Because Drancourt merely summarizes Schoedon, and because the cited Schoedon culture attempts were in a monocyte medium, Drancourt does not teach or suggest that the culture is not a cell culture in monocyte cells, as recited in claim 30. Thus Drancourt does not satisfy the limitations of claim 30, and the §102(b) rejection of that claim over Drancourt should also be reversed for that reason, as well as for its dependency on claim 1.

e. Claim 31

Because Drancourt merely summarizes Schoedon, and because the cited Schoedon culture attempts were in a monocyte medium, Drancourt does not teach or suggest that the culture is a cell culture in immortalized cells other than monocyte cells, as recited in claim 31. Thus Drancourt does not satisfy the limitations of claim 31, and the §102(b) rejection of that claim over Drancourt should also be reversed for that reason, as well as for its dependency on claim 1.

f. Conclusion

For at least the foregoing reasons, Drancourt does not teach or suggest all of the features recited in any of claims 1, 30, 31, 68 and 69. Accordingly, the §102(b) rejection of those claims over Drancourt should be reversed.

C. Claims 1, 11, 15, 25, 29-32, 35-42, 63 and 66-69 Would Not have been Obvious over Muller, Schoedon or Drancourt in View of Kent and Harlow and Lane

Claims 1, 11, 15, 25, 29-32, 35-42, 63 and 66-69 are rejected as having been obvious under 35 U.S.C. §103(a) over Muller, Schoedon or Drancourt in view of Kent, Arch. Pathol. Lab. Med. 104 (10), pp. 544-47 (1980) and Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory 1988, Chapters 14/5/6.

1. Claims 1, 30, 31, 68 and 69

The subject matter of claims 1, 30, 31, 68 and 69 is not taught or suggested by any of Muller, Schoedon or Drancourt for all of the separately argued reasons discussed above.

Kent and Harlow and Lane do not remedy the deficiencies of Muller, Schoedon and Drancourt. In particular, Kent and Harlow and Lane also do not teach or suggest any culture comprising a culture medium and a bacterium responsible for Whipple's disease, or thus the limitations on such cultures imposed by the subject claims. Accordingly, the §103 rejection of claims 1, 30, 31, 68 and 69 should be reversed.

2. Claims 11, 15, 30-32, 35-42, 66 and 67

These claims depend from claim 1 and thus would not have been obvious over the cited references, and thus the rejection should be reversed, for at least the same reasons as claim 1.

3. Claims 32 and 37

None of the applied references teach or suggest culturing *Tropheryma whippelii* in fibroblast cells, as required by claims 32 and 37. The Final Rejection has provided no basis for suggesting that this subject matter would have been obvious other than a passing reference

to "... immortalized cell lines such as fibroblasts as taught by Muller et al 1999 or Drancourt 1999 ..." (Final Rejection, page 8, line 29). However, neither of those references even mentions fibroblasts. Thus the §103 rejection of these claims should be reversed.

4. Claims 36 and 37

Claims 36 and 37 include the limitations of both claims 1 and 68. Thus claims 36 and 37 would not have been obvious for at least the reasons discussed herein relating to each of those claims, as well as the separate reasons for claim 37 discussed above. Accordingly, the §103 rejection of claims 36 and 37 should be reversed.

5. Claim 42

Claim 42 recites a culture medium that does not comprise monocyte cells. As noted above, the media of the primary references include monocyte cells, and the secondary references do not disclose or suggest culture media for *Tropheryma whippelii*. Thus the subject matter of claim 42 would not have been obvious from the cited references, and the §103 rejection of that claim should be reversed.

6. Claim 63

Claim 63 includes the subject mater of claim 1, and thus would not have been obvious over the cited references, and thus the rejection should be reversed, for at least the same reasons as claim 1. In addition, as is the case with claims 43-45, which were not included in the §103 rejection, none of the cited references teaches or suggests the deposited strain recited in claim 63. Thus the subject matter of claim 63 would not have been obvious from the cited references, and the §103 rejection of that claim should be reversed.

7. Claims 10, 25 and 29

None of the applied references teach or suggest (1) an antigen isolated from the *Tropheryma whippelii* bacterium in the culture according to claim 1, as recited in claim 10, or (2) a method for the in vitro diagnosis of diseases associated with infections caused by

Tropheryma whippelii, comprising contacting serum or any other biological fluid of a patient with the antigen of claim 10, and detecting an immunological reaction, as recited in claims 25 and 29.

The Office Action relies for support for the §103 rejection on a passing reference to "the readily available bacteria." However, because the art had not discovered how to make a culture in accordance with claim 1, there was no such "readily available" *Tropheryma whippelii* bacteria. While that bacteria could be identified in patient samples and materials infected with it, it could not be produced in amounts necessary to produce the claimed isolated antigen or to practice the claimed methods using that antigen. Thus, it would not have been obvious to produce such antigen or to practice such methods. Accordingly, the §103 rejection of these claims should be reversed.

8. Claims 11 and 15

None of the applied references teach or suggest a method for the in vitro diagnosis of diseases associated with infection cased by *Tropheryma whippelii*, comprising contacting serum or any other biological fluid of a patient with a culture according to claim 1 or a *Tropheryma whippelii* bacterium obtained from such culture, and detecting an immunological reaction, as recited in claims 11 and 15. Again, the Office Action relies for support for the §103 rejection on a passing reference to "the readily available bacteria." However, because the art had not discovered how to make a culture in accordance with claim 1, there was no such "readily available" *Tropheryma whippelii* bacteria. While *Tropheryma whippelii* bacteria could be identified in patient samples and materials infected with it, it could not be produced in amounts necessary to practice the claimed methods, which rely on the existence of the culture of claim 1. Nor was the culture of claim 1 available in the prior art for use in such methods. Thus, it would not have been obvious to practice such methods. Accordingly, the §103 rejection of these claims should be reversed.

9. Secondary Considerations Evidence

The record is replete with evidence that the claimed invention not only would not have been obvious, but that it was not obvious to those of at least ordinary skill in the art. The record shows the existence of a very longfelt unsatisfied need for the claimed invention, which others had tried and failed to achieve, that was first met by Appellants by way of the claimed invention, resulting in much acclaim in the industry.

In Eli Lilly Co. v. Zenith Goldline Pharmaceuticals, Inc., 471 F.3d 1369 (Fed Cir. 2006), the Federal Circuit held that establishing four of the five secondary considerations, including a long-felt need, failure of others, industry acclaim and unexpected results, strongly confirmed that the compounds that were the subject of dispute were not obvious in view of the prior art references. Similarly, the numerous articles presented to the Patent Office address many of the same "secondary considerations" exhibited by the presently claimed invention.

For example, Relman at page 752, first sentence, stated that "Cultivation of the bacillus associated with Whipple's disease, *Tropheryma whippelii*, has been an elusive goal for many generations of clinicians and microbiologists familiar with this disease." Maiwald at page 801, second column, confirmed that "the cultivation of this bacterium has been a goal of clinicians for several decades." Even the Drancourt reference applied in the Office Action refers to "90 years of isolation attempts" at page 9 of the translation. Maiwald confirms that "*..many attempts have been made to cultivate this bacterium in vitro. It was eventually isolated, in 2000....*" (see Maiwald, citing to Raoult et al., the inventors of the present application), and Bentley similarly confirms that "i*solation of the bacterium Tropheryma Whipplei was achieved in 2000, in a long term culture system with human fibroblasts, with a reported generation time of 18 days*"(see page 637 column 2 of Bentley). See paragraph 13 of the Drancourt Declaration. Maiwald even noted that Schoedon's teachings did not do so

and were not reproducible. See page 802, first column of Maiwald. See also, paragraph 4 of the Drancourt Declaration. In contrast, the unexpected successful culture of *Tropheryma whippelii* bacterium according to the culture condition taught by Raoult et al. (the inventors of the present application) have been confirmed by Relman's team. See paragraph 6 of the Drancourt Declaration. Thus the objective evidence of record is clear and convincing "secondary considerations" evidence of the non-obviousness of the claimed invention, and the §103 rejection should be reversed in view of that evidence.

10. Conclusion

For at least the foregoing reasons, Appellants submit that Muller, Schoedon, Drancourt, Kent, and Harlow and Lane, in combination or alone, do not teach or suggest the subject matter of claims 1, 11, 15, 25, 29-32, 35-42, 63 or 66-69. Thus the §103 rejection of those claims should be reversed.

VIII. CONCLUSION

For all of the reasons discussed above, it is respectfully submitted that the rejections are in error and that claims 1, 10, 11, 15, 25, 29-32, 35-42, 63 and 66-69 are in condition for allowance. For all of the above reasons, Appellants respectfully request this Honorable Board to reverse the rejections of claims 1, 10, 11, 15, 25, 29-32, 35-42, 63 and 66-69.

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APPENDIX A - CLAIMS APPENDIX

CLAIMS INVOLVED IN THE APPEAL:

1. A culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days as detected by inverted microscopy, wherein the bacterium is *Tropheryma whippelii*.

10. An antigen isolated from the *Tropheryma whippelii* bacterium in the culture according to claim 1, wherein said antigen is a protein of 200 kD determined by polyacrylamide gel electrophoresis using the Western blotting technique, which reacts with a specific monoclonal antibody directed against the bacterium *Tropheryma whippelii* responsible for Whipple's disease or an antigen of said bacterium, said antibody being produced by a hybridoma deposited in the CNCM of the Institut Pasteur under the Deposit No. I-2411.

11. A method for the *in vitro* diagnosis of diseases associated with infections caused by *Tropheryma whippelii*, comprising contacting serum or any other biological fluid of a patient with a culture according to claim 1 or a *Tropheryma whippelii* bacterium obtained from said culture, and detecting an immunological reaction.

15. A method for the *in vitro* diagnosis according to claim 11, comprising:

- depositing a solution containing said *Tropheryma whippelii* bacterium in or on a solid support;
- introducing serum or any other biological fluid into or onto said support;
- introducing a solution of a labeled antibody specific for a human immunoglobulin, which recognizes said bacterium, into or onto the support;
- observing an incubation period;

- rinsing the solid support; and
- detecting an immunological reaction.

25. A method for the *in vitro* diagnosis of diseases associated with infections caused by *Tropheryma whippelii*, comprising contacting serum or any other biological fluid of a patient with the antigen of claim 10, and detecting an immunological reaction.

29. A method for the *in vitro* serological diagnosis according to claim 25, comprising:

- depositing a solution containing said antigen in or on a solid support;
- introducing serum or any other biological fluid into or onto said support;
- introducing a solution of a labeled antibody specific for a human immunoglobulin, which recognizes said antigen, into or onto the support;
- observing an incubation period;
- rinsing the solid support; and
- detecting an immunological reaction.

30. A culture according to claim 1, wherein said culture is not a cell culture in monocyte cells.

31. A culture according to claim 1, wherein said culture is a cell culture in immortalized cells other than monocyte cells.

32. A culture according to claim 31, wherein the immortalized cells are fibroblast cells.

35. A culture according to claim 1, wherein the bacterium is in a cell in the culture medium.

36. A culture according to claim 35, wherein the cell has a dividing time greater than the doubling time of the bacterium.

37. A culture according to claim 36, wherein the cell is a fibroblast cell.

38. A culture according to claim 1, wherein the bacterium is capable of reproducibly and detectably multiplying over time in said culture medium through successive subcultures.

39. A culture according to claim 1, wherein the bacterium has been established in culture through successive subcultures.

40. A culture according to claim 1, wherein the bacterium is of the same species as the *Tropheryma whippelii* bacterium strain deposited in the CNCM of the Institut Pasteur under Deposit No. I-2202.

41. A culture according to claim 1, wherein the bacterium comprises a rpoB gene comprising a partial sequence amplifiable by primers of a sequence identical to SEQ ID NO:4 or 5.

42. A culture according to claim 41, wherein said culture medium does not comprise monocyte cells.

63. A culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture such that the bacterium can reproducibly and detectably multiply over time in the culture medium for at least 72 days through successive subcultures, as detected by inverted microscopy,

wherein the bacterium is of the *Tropheryma whippelii* bacterium strain deposited in the CNCM of the Institut Pasteur under Deposit No. I-2202,

wherein the bacterium comprises a rpoB gene comprising a partial sequence amplifiable by primers of a sequence identical to SEQ ID NO:4 or 5.

66. A culture according to claim 1, wherein the bacterium is capable of reproducibly and detectibly multiplying over time in a culture medium comprising fibroblast cells.

67. A culture according to claim 1, wherein the bacterium can reproducibly and detectably multiply over time in the culture medium for 72 days.

68. A culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and said cell has a dividing time greater than the doubling time of the bacterium.

69. A culture comprising a culture medium and a bacterium responsible for Whipple's disease, said bacterium being isolated and established in culture in a cell in the culture medium, wherein the bacterium is *Tropheryma whippelii*, and the cell is selected such that it does not multiply so rapidly relative to the growth of the bacterium as to cause a dilution effect of the bacterium.

APPENDIX B - EVIDENCE APPENDIX

A copy of each of the following items of evidence relied on by the Appellant is attached:

1) Maiwald, et al., "Cultivation of *Tropheryma whipplei* from Cerebrospinal Fluid," Journal of Infectious Disease, Vol. 188, pp. 801-808 (September 15, 2003) (submitted with the Amendment filed on February 5, 2007);

2) Helin, et al., "Measles Virus Replication in Cells of Myelomonocytic Lineage is Dependent on Cellular Differentiation Stage," Virology, Vol. 253, pp. 35-42 (1999) (submitted with the Amendment filed on February 5, 2007);

3) Abstract of Foa, et al., "Growth Pattern of the Human Promyelocytic Leukemia Cell Line HL60," Cell Tissue Kinet., Vol. 15, No. 4, pp. 399-404 (July 1982) (submitted with the Amendment filed on February 5, 2007);

4) Bentley, et al., "Sequencing and Analysis of the Genome of the Whipple's Disease Bacterium *Tropheryma whipplei*," The Lancet, Vol. 361, pp. 637-644 (February 22, 2003) (submitted with the Amendment filed on February 5, 2007);

5) Relman, "Editorial: The Whipple Bacillus Lives (Ex Vivo)," The Journal of Infectious Diseases, Vol. 176, pp. 752-754 (1997) (submitted with the Amendment filed on February 5, 2007);

6) Hinrikson et al., "Detection of Three Different Types of 'Tropheryma Whippelii' Directly from Clinical Specimens by Sequencing, Single-Strand Conformation Polymorphism (SSCP) Analysis and Type-Specific PCR of Their 16S-23S Ribosomal Intergenic Spacer Region," International Journal of Systematic Bacteriology, Vol. 49, pp. 1701-1706 (October 1999);

7) Raoult et al., The New England Journal of Medicine, Vol. 342, No. 9, pp. 620-625 (March 2, 2000); and

8) Drancourt Declaration Under 37 CFR §1.132 originally submitted September 9, 2005.

MAJOR ARTICLE

Cultivation of *Tropheryma whipplei* from Cerebrospinal Fluid

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(See the editorial by Scheld on pages 797–800.)

Whipple disease (WD) is a systemic disorder caused by the bacterium *Tropheryma whipplei*. Since the recognition of a bacterial etiology in 1961, many attempts have been made to cultivate this bacterium in vitro. It was eventually isolated, in 2000, from an infected heart valve, in coculture with human fibroblasts. Here we report the isolation of 2 new strains of *T. whipplei* from cerebrospinal fluid (CSF) of 2 patients with intestinal WD but no neurological signs or symptoms. One culture-positive specimen was obtained before treatment; the other was obtained 12 months after discontinuation of therapy, at a time of intestinal remission. In both cases, 15 passages of the cultures were completed over 17 months. Bacterial growth was measured by quantitative polymerase chain reaction, which suggested a generation time of 4 days. Staining with YO-PRO nucleic-acid dye showed characteristic rod-shaped bacteria arranged in chains. Fluorescent in situ hybridization with a *T. whipplei*–specific oligonucleotide probe, a broad-range bacterial probe, and a nonspecific nucleic-acid stain indicated that all visible bacteria were *T. whipplei*. Scanning electron microscopy and transmission electron microscopy showed both intracellular and extracellular bacteria. This first isolation of *T. whipplei* from CSF provides clear evidence of viable bacteria in the central nervous system in individuals with WD, even after prolonged antibiotic therapy.

In 1907, George H. Whipple described the postmortem examination of a patient who had died of a chronic disease presenting with arthritis, fever, weight loss, and cough [1]. He observed deposits of fat and fatty acids in the intestinal mucosa and mesenteric lymph nodes and named the disease "intestinal lipodystrophy." Whipple also observed small bacteria in silver-stained sections of a mesenteric lymph node, but he did not interpret this finding as causally related to the disease. Subsequent reports characterized Whipple disease (WD) as a rare, chronic, systemic disease, involving predominantly the intestinal tract but also a variety of other organs, especially the central nervous system (CNS) [2]. The etiology remained unclear for >40 years, until a bacterial cause was suggested by 2 observations: (1) a 1952 report of successful antibiotic treatment [3], and (2) the 1961 detection, by electron microscopy, of numerous, small, uniform bacteria in affected tissues [4, 5]. Both types of observations were subsequently confirmed and extended by many others.

Numerous attempts have been made to cultivate the WD bacterium in the laboratory, but they have either failed or yielded results that proved erroneous [2]. *Streptococcus* species, *Corynebacterium* species, and *Haemophilus* species are among the organisms so implicated [2]. Cultivation of this bacterium has therefore been a goal of clinicians and microbiologists for several decades. Characterization of the WD bacterium at the molecular level was accomplished during the early 1990s, by polymerase chain reaction (PCR) using broad-range primers

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to analyze bacterial 16S rDNA [6, 7]; analysis of the novel sequence established a phylogenetic relationship to the actinomycetes, and the name "*Tropheryma whippelii*" was proposed [7]. In 1997, on the basis of the notion that macrophages are the cell type most prominently involved in the pathology of WD, investigators inoculated interleukin-4–deactivated macrophages with heart-valve tissue affected by WD and reported propagation of bacteria [8]. However, this finding could not be confirmed in subsequent studies [9].

Long-term cocultivation of the WD bacterium with a human fibroblast cell line inoculated with heart-valve tissue was reported by Raoult et al. in 2000 [10]. The infection status of the fibroblasts was determined by microscopy, by periodic-acid–Schiff (PAS) staining, and by immunofluorescence, with the patient's serum. After inoculation of 1 cm^2 of cell monolayer, the cultures were expanded to 3750 cm^2 of infected cells over 7 passages within 9 months. After each passage, qualitative PCR detected DNA of the WD bacterium. The estimated bacterial doubling time was 18 days, which is longer than that of any other characterized bacterium. A second strain was subsequently isolated from a duodenal biopsy specimen [11], and the species designation was modified to "*whipplei*" [12]. Taken together, these reports provide good evidence for in vitro propagation of *T. whipplei*. Nonetheless, two important types of data are missing: (1) quantitative assessment of bacterial growth in vitro, by a molecular method, and (2) physical association of the *T. whipplei* 16S rRNA sequence with cultivated bacterial cells, by fluorescent in situ hybridization (FISH). The latter has been proposed as an important link between bacterial sequence and visible cells, especially when new taxa are described [13, 14]. Furthermore, the presence of viable *T. whipplei* bacteria has not been established in the CNS of individuals with WD. The availability of two cerebrospinal fluid (CSF) samples with large numbers of WD bacteria provided an opportunity to isolate new strains of *T. whipplei* and address all of these important issues.

PATIENTS, MATERIALS, AND METHODS

Patients and specimens. This work was approved by the Stanford University Administrative Panel on Human Subjects in Medical Research. CSF from 2 patients was used in these cultivation studies. Both patients presented with intestinal WD that was diagnosed by histopathology and by PCR analysis of *T. whipplei* 16S rDNA. Case 1 was a 74-year-old German man; the CSF specimen was obtained for the purpose of staging, before the initiation of therapy. The patient had no neurological symptoms or signs. Case 2 was a 52-year-old German woman; staging examinations by PCR analysis of *T. whipplei* in CSF [15] revealed CNS infection, but the patient had no neurological symptoms or signs. The patient was treated with an initial course of 2 weeks of penicillin plus streptomycin, followed by 1 year of oral cotrimoxazole. The CSF specimen used for culture was obtained for the purpose of monitoring response to therapy, 24 months after diagnosis and 12 months after discontinuation of antibiotics. At that time, results of PCR analysis of *T. whipplei* 16S rDNA of duodenal tissue were negative, and histology showed remission, in accordance with published criteria [16]. Diagnostic PCR analysis of *T. whipplei* 16S rDNA [15] showed strongly positive results for the CSF specimens from both patients, and the amplified sequence was completely homologous to the *T. whipplei* 16S rDNA (GenBank accession number X99636).

Cultivation methods. Cell cultivation on human fibroblasts was performed essentially as described elsewhere [10–12], with the following modifications: HEPES buffer (12.5 mM) was used in the medium, and fetal-calf-serum content was reduced from 10% to 1%, after confluent cell monolayers were obtained and before inoculation with bacteria. MRC-5 primary human embryonic lung fibroblasts (CCL-171; American Type Culture Collection) were cultivated in 25-cm^2 tissue-culture flasks (5-mL medium) and were inoculated with 500 μL of original CSF. Initial passages of the cultures were performed in 25-cm^2 flasks; 75-cm^2 flasks (25 mL) and 150-cm^2 flasks (35 mL) were later used for large-scale cultures. Each passage of the cultures involved inoculation of 20%–25% of the volume of supernatant onto new fibroblast monolayers after 4–6 weeks of incubation. Medium was changed infrequently: during the first passage, the medium was changed only after 3 weeks, and, during subsequent passages, the medium was either not changed or changed only after ~4 weeks of incubation. Beginning with the 13th passage, both MRC-5 cells and primary human foreskin fibroblasts (a gift from E. S. Mocarski, Stanford University) were used in parallel, for cultivation.

For quantitative measurement of bacterial growth, cell monolayers were cultivated in 6-well tissue-culture plates (9.5 cm^2/well) containing 2 mL of medium. On day 0, duplicate wells were inoculated with 0.5 mL of vigorously vortexed culture supernatant from a flask containing infected material. The contents of these wells were harvested on days 1 and 28 after inoculation: first, 1.25 mL of culture supernatant was removed, and then the cell monolayer was removed by a cell scraper and was harvested together with the residual 1.25 mL of supernatant. Both portions were frozen (−80°C) before analysis.

PCR. Tissue-culture supernatant or cell monolayers were centrifuged (18,000 *g* for 10 min), and DNA from the pellet was extracted as described elsewhere [15, 17]. To detect the presence of *T. whipplei* 16S rDNA, qualitative PCR using primers whip1 and whip2 [17] was performed; for bacterial identification, PCR using broad-range primers 8FPL plus 806R and 515FPL plus 1492RPL to analyze bacterial 16S rDNA [18]. Quantitative competitive PCR was performed according to

published protocols [19] and used the primers whip1 and whip2 [17] and a synthetic internal-standard molecule. This molecule (the "mimic") was constructed by PCR, according to instructions from the Clontech PCR mimic-construction kit. Composite primers were designed on the basis of the sequence of the *Bordetella bronchiseptica* filamentous hemagglutinin gene, *fhaB* [20], and *T. whipplei* 16S rDNA, so that the mimic consisted of a 217-bp sequence including whip1 and whip2 primer sequences at its ends. As a result, the mimic was easily distinguished, on the basis of size, from the 267-bp 16S rDNA amplification product (the "target") of *T. whipplei*. The product from *T. whipplei* and the mimic were each cloned into the TA vector (Invitrogen), plasmid DNA was extracted and quantified, and stock solutions containing 10^8 copies of each plasmid molecule/μL were prepared. Serial dilutions of the mimic molecule were used as internal standards in the PCRs, and serial dilutions of the *T. whipplei* product were used as quantitative references in control reactions. Samples from culture were initially tested against 10-fold dilutions of the "mimic," and then, for more accurate measurement, against 2-fold dilutions. The mimic concentration that, in agarose-gel electrophoresis, gave DNA-band intensity equal to that of the *T. whipplei* product was used to estimate the number of copies of *T. whipplei* rDNA in the sample.

Nucleic-acid staining. Nucleic acids in cultivated material were stained directly with YO-PRO-1 fluorescent dye (Molecular Probes). Culture supernatant was fixed in 3.7% formaldehyde, spotted onto glass slides, and air-dried. The slides were then overlaid with 2 μM YO-PRO-1 in water, incubated for 15 min, rinsed with water, immersed for 15 min in water, rinsed again, air-dried, and mounted with Vectashield mounting fluid (Vector Laboratories) and a coverslip (all steps were performed in the dark).

FISH. FISH was performed essentially as described elsewhere [21], with some modifications. In brief, culture supernatant was centrifuged (10,000 *g* for 10 min), and the pellets were resuspended in 1 × PBS, mixed with an equal volume of ethanol (final concentration, 50%), spotted onto Teflon-coated 10-well slides (Erie Scientific), and air-dried, at 45°C, on the wells. The samples on the slides were then fixed by incubations of 3 min each in 50%, 80%, and 96% ethanol. Hybridization was performed for 2 h at 46°C, with a solution containing 5 × SET, 1% SDS, 10% dextran, 0.2% bovine serum albumin, 0.1 mg polyadenosine/mL, and 5 μg of labeled probe/mL. The slides were then washed 3 times, for 10 min at 46°C, with 0.2 × SET at 46°C, rinsed with water, stained with 1 μM YO-PRO-1 in 1 × SET as described above, rinsed again, and mounted with Vectashield and coverslips. The following oligonucleotide probes were used: the *T. whipplei*–specific probe Tw16S-652 (5′-TTCCGCTCTCCCCTATCGCACTCT), the negative-control probe Tw16S-Cnt (5′-AAGGCGAGAGGGGATAGCGTGAGA) [21], the broad-range bacterial probe Eub16S-338 (5′-GCTGCC-TCCCGTAGGAGT) [22], and the probe HGC69a (5′-TATAGT-TACCACCGCCGT) for gram-positive bacteria with high G+C content [23]. Tw16S-652, Tw16S-Cnt, and HGC69a were labeled with the fluorophore Cy-3, and probe Eub16S-338 was labeled with Cy-5. Cultures of "*Corynebacterium aquaticum*" (ATCC 14665), *Cellulomonas cellulans* (ATCC 27402), and *Agromyces ramosus* (ATCC 25173)—all *Actinobacteria*—were used as bacterial controls. Slides were viewed and images were recorded by use of a BioRad MRC-1024 Laser Scanning Confocal Imaging System, as described elsewhere [21].

Electron microscopy. Cell monolayers were cultivated on round, 18-mm glass coverslips in 12-well (4-cm^2) tissue-culture plates. Four weeks after inoculation, the medium was removed, and the cells were fixed, for 2 days, with 1.5% glutaraldehyde that was buffered to pH 7.3 by sodium cacodylate and that was made isotonic by the addition of sucrose. For scanning electron microscopy (SEM), the coverslips with cells and bacteria were dehydrated with alcohol and a critical-point bomb, were sputter-coated with 100-Å gold, and then were examined by use of an Hitachi S-2400 scanning electron microscope operating at an accelerating voltage of 15 kV. For transmission electron microscopy (TEM), the monolayers were postfixed, for 1 h, in 2% buffered osmic acid, dehydrated with alcohol, and embedded in epoxy resin. Sections were cut at 50-nm thickness, were stained serially with uranyl acetate and lead hydroxide, and then were examined by use of a Phillips 200 electron microscope operating at an accelerating voltage of 75 kV.

Strain deposition. The isolate from patient 2 (strain TW08/27) has been deposited in the American Type Culture Collection (ATCC culture number pending).

RESULTS

Six weeks after inoculation of MRC-5 primary human embryonic lung fibroblast monolayers with CSF from cases 1 and 2, qualitative PCR used to test for *T. whipplei* in culture supernatants from the 2 infected monolayers gave positive results. Cellular and bacterial material from 5 mL of supernatant was then concentrated, by centrifugation, in 1 mL and then was inoculated onto fresh monolayers in 25-cm^2 flasks. On days 1 and 15 after this passage, 100 μL of supernatant was collected and analyzed by quantitative PCR. A "low-resolution" quantitative-PCR analysis (using 10-fold dilutions of the mimic) indicated an increase in rDNA copy number, from 10^5/mL (CSF of case 1) and $<10^5$/mL (CSF of case 2) on day 1 to $\geq 10^6$/mL (in both cases) on day 15. Before inoculation, the original CSF specimens had shown copy numbers of 10^4/mL (case 1) and $<10^4$/mL (case 2), by the same PCR. In the subsequent, similar passage, supernatant from both cultures was stained with YO-PRO and showed small, rod-shaped bacteria in a characteristic

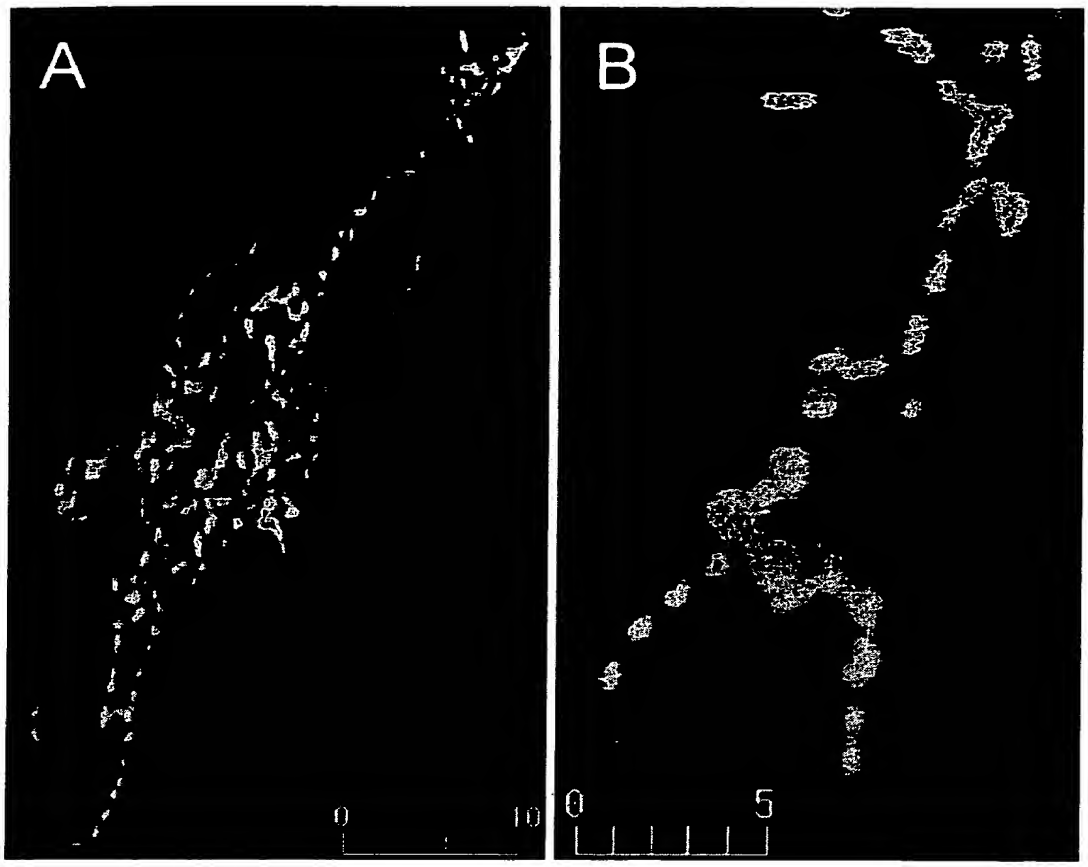

Figure 1. Photomicrograph of culture supernatant stained with YO-PRO nucleic-acids dye. *A*, Strain TW09/02 in third passage (original magnification, ×3000). *B*, Strain TW08/27 in ninth passage (original magnification, ×5000). Scale bars represent micrometers.

chainlike arrangement (figure 1*A*). Culture supernatant was also examined by PCR using broad-range primers to analyze bacterial 16S rDNA, in an assay that targets a 1443-bp region of the 16S rDNA. Direct sequencing of PCR products revealed unambiguous readings; the sequence from case 1 was a perfect match to that of *T. whipplei* [24]; the products from case 2 had only 2 nucleotide mismatches, in positions where they would not affect the 16S rRNA structure.

A total of 15 passages were performed with both cultures, over a period of 17 months. Beginning with the 13th passage, human foreskin fibroblasts were used in parallel with MRC-5 cells, because they appeared to form more-coherent monolayers and remained morphologically unaltered over longer incubation times. Cultures were regularly checked for the presence of bacteria, by staining the supernatant with YO-PRO; this was done at each passage, usually between the fourth week of incubation and the time of transfer to a new cell monolayer, and showed characteristic-looking bacteria (figure 1*B*). The strain from case 1 was designated "TW09/02," the strain from case 2 "TW08/27." The cultures were expanded to 40 flasks (150 cm^2 each) for strain TW09/02 and to 60 flasks for strain TW08/27. PCR analysis of broad-range bacterial 16S rDNA was repeated with both strains after their 15th passage, with the same results. Material from the 60 flasks with strain TW08/27 was harvested, and bacterial DNA was extracted and used for a genome-sequencing project [25] (see the http://www.sanger.ac.uk/Projects/T_whipplei/ Web site).

Quantitative-PCR studies (see Patients, Materials, and Methods) were performed again after the 11th passage, using 10-fold and, subsequently, 2-fold dilutions of the mimic (figure 2). Data from supernatants and data from combined fractions (supernatant plus cell monolayer) harvested on days 1 and 28 after inoculation were compared (table 1). *T. whipplei* 16S rDNA copy numbers were ~100-fold greater on day 28 than they were on day 1. In addition, rDNA copy numbers in the combined fractions were ~10-fold greater than those measured in the supernatants alone. These data suggest that, on average, the bacteria have completed 7 divisions during the intervening

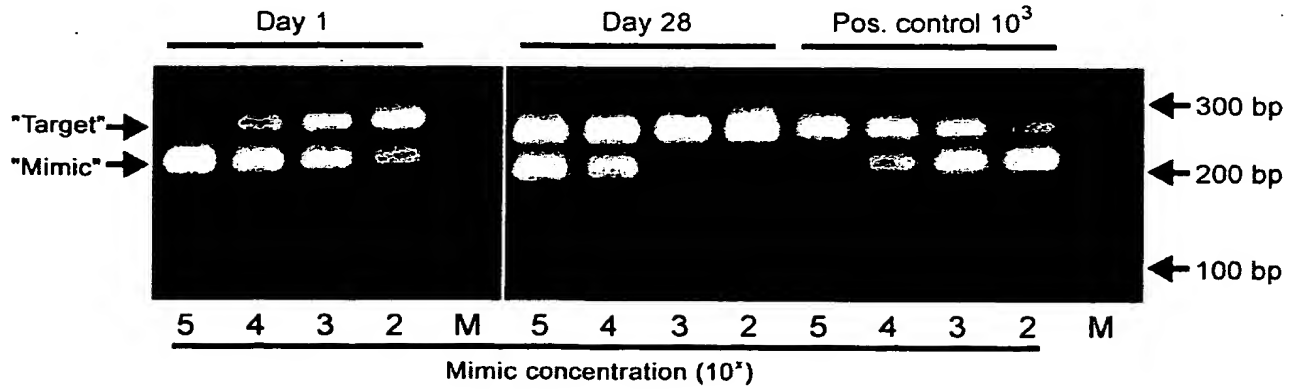

Figure 2. Agarose gel showing results of polymerase chain reaction (PCR) in cultures in 6-well plates (see Patients, Materials, and Methods), for combined fractions (supernatant plus cell monolayer) from strain TW08/27, on days 1 and 28 of incubation, tested against initial 10-fold dilutions of the "mimic" molecule. A positive control with the cloned *Tropheryma whipplei* "target" (10^3 copies) was also used. The copy numbers of mimics and targets used in a 50-μL PCR are given. Because of dilution factors, the calculated copy number per milliliter of culture material (table 1) is 1.5 log higher than the copy number used in a 50-μL PCR. M, molecular-weight marker.

27 days, which corresponds to a bacterial generation time of ~4 days. To confirm the specificity of the quantitative-PCR results, a target band from this assay was sequenced, for both strains; this sequence was identical to the 16S rRNA sequence of *T. whipplei*.

For both strains, FISH experiments with culture supernatant were performed after the 12th and 15th passages. All visible bacteria in supernatants hybridized with the *T. whipplei*–specific probe Tw16S-652, the broad-range bacterial probe Eub16S-338, and the actinobacterial probe HGC69a but not with the negative-control probe Tw16S-Cnt. All bacterial control strains hybridized with Eub16S-338 and HGC69a, none hybridized with Tw16S-Cnt, and only "*C. aquaticum*" hybridized, very faintly, with Tw16S-652, as described elsewhere [21]; this faint signal was easily distinguishable from the much-brighter signal in the 2 CSF cultures. Triple-label experiments, with YO-PRO, Tw16S-652, and Eub16S-338, revealed colocalized staining patterns with the 3 labels, for all bacteria in both cultures (figure 3), indicating a homogenous population of *(T. whipplei)* bacteria.

Electron microscopy of culture material from both strains was performed after the 14th passage. SEM showed intact extracellular bacteria (figure 4*A*), and TEM showed well-preserved bacteria both in extracellular locations and within the cytoplasm of healthy-appearing fibroblasts (figure 4*B*).

DISCUSSION

The results of the present study indicate that viable *T. whipplei* strains are found in the CSF of patients with WD and that they can be propagated in the presence of human fibroblasts in culture. These data confirm and expand on the findings reported by Raoult et al. [10–12]. They also provide the first quantitative measurement of the growth of *T. whipplei* in vitro. A previous report [8], describing the growth of *T. whipplei* in interleukin-4–deactivated macrophages, has been not confirmed, either by us (M.M. and D.A.R., unpublished results) or by other investigators [9].

Our data also document the first cultivation of *T. whipplei* from CSF samples. CSF is ideally suited for such studies, since it is a relatively simple fluid that is normally sterile. The examination of CSF has special relevance for diagnostic testing for WD, because (1) bacteria appear to invade the CNS early in the disease and (2) late manifestations affecting the CNS pose a significant threat to patients [2, 15]. This is illustrated by a number of published cases with symptomatic CNS disease, cases in which bacteria appeared to have been eradicated from the intestinal mucosa after therapy [2, 15, 17, 26–29]. One noteworthy case presented with severe insomnia as the only symptom 8 years after intestinal WD had been diagnosed and treated; at that time, results of intestinal/histological examination and PCR analysis of intestinal tissue were negative but PCR analysis showed that CSF was positive for *T. whipplei* [29].

Table 1. Results of quantitative polymerase chain reaction, for the 2 *Tropheryma whipplei* strains from cultures in 6-well plates (see Patients, Materials, and Methods).

Day after inoculation	Strain TW09/02		Strain TW08/27	
	Supernatant	Combined fraction	Supernatant	Combined fraction
1	5×10^3/mL	5×10^4/mL	$<5 \times 10^3$/mL	2×10^4/mL
28	8×10^5/mL	8×10^6/mL	5×10^5/mL	5×10^6/mL

NOTE. Both the culture supernatants (1.25 mL) and the combined fractions (1.25 mL), the latter of which consisted of supernatant plus cell monolayer, were tested during the 11th passage on each of the 2 days.

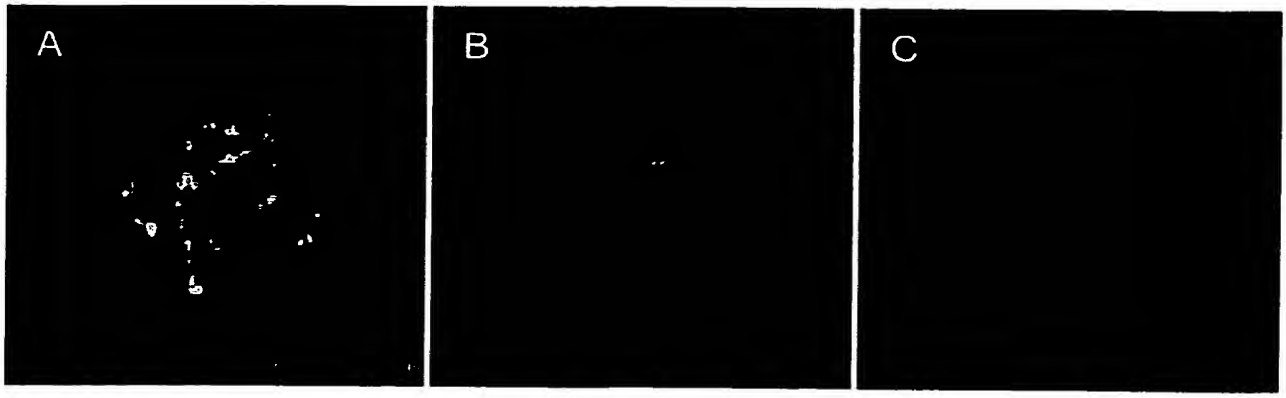

Figure 3. Photomicrographs after fluorescent in situ hybridization with strain TW08/27 from the 14th passage of the cultures, for dual hybridization with probes Tw16S-652 and Eub16S-338, followed by YO-PRO stain. *A*, YO-PRO stain (nonspecific DNA stain) viewed with the fluorescein isothiocyanate channel. *B*, Probe Tw16S-652 (*Tropheryma whipplei* specific) viewed with the Texas Red channel. *C*, Probe Eub16S-338 (bacterial broad range) viewed with the Cy-5 channel (original magnification, ×2000). Scale bar represents micrometers.

Often, manifestations of *T. whipplei* in CNS respond only partially to antibiotics and have a poor prognosis.

A previous study examined CSF samples from 24 patients with WD that were obtained at various times before and after therapy [15]; even in neurologically asymptomatic patients, PCR results were positive for the presence of *T. whipplei* in 7 of 10 cases before therapy and in 3 of 11 cases after therapy. These data indicate that the bacterium or its components are commonly present in the CNS of patients with intestinal WD and that, even in the presence of prolonged therapy with antibiotics, bacterial clearance may be delayed or uncertain. Furthermore, the data underscore the importance of using antibiotics that cross the blood-brain barrier. The isolation of 2 *T. whipplei* strains from CSF supports these concepts and emphasizes the importance of PCR-based, sensitive approaches for the detection and monitoring of CNS infection. The present study provides new evidence of viable *T. whipplei* in the CNS of patients with WD, even in the absence of neurological symptoms, and demonstrates that the bacterium can persist in a viable state, even after 1 year of therapy and intestinal-disease remission.

Quantitative measurement of bacterial growth is an important contribution to the evolving story of the propagation of *T. whipplei* ex vivo. The use of an internal standard (i.e., a mimic) avoids the potential problems of other types of PCR assays, in which PCR inhibitors might interfere with quantification [19]. Our calculated doubling time of 4 days differs from the previously reported time of 18 days, which was based on semiquantitative microscopic assessment of inclusions in fibroblast monolayers, inclusions that were shown to be positive for *T. whipplei* [10] when the PAS reagent was used, but it is still among the longest observed doubling times for any bacteria. This difference might be due either to the different measurement methods or culture conditions or to the differences between *T. whipplei* strains. Knowledge of the generation time is clinically relevant; with a doubling time of 4 days, a typical, 14-d intravenous therapy–induction period [30] spans only 3 replication cycles and thus might have to be reconsidered.

Bacterial morphology and the chainlike arrangement were distinctive when revealed by YO-PRO staining (figure 1). FISH now integrates, for the first time, bacterial morphology and the 16S rRNA sequence of *T. whipplei*. A previous study with sections from intestinal biopsy specimens did not resolve individual bacteria, probably because of high bacterial density and the thickness of the sections [21]. Triple-label experiments in the present study (figure 3) showed that nonspecific staining of DNA by YO-PRO, a broad-range bacterial probe, and a WD-specific probe all colocalized to the same bacterial shapes. These data and the absence of ambiguities in the PCR-based analysis of broad-range bacterial 16S rDNA performed during the third and 15th passages indicate that the cultures were not contaminated with other bacteria. Multiple FISH experiments clearly showed small, rod-shaped bacteria, but the slender shapes and the chainlike arrangement were not as well preserved as were those seen in staining by YO-PRO. The different morphologies seen by these 2 methods may arise from the different fixation procedures (i.e., formalin vs. alcohol) and/or the additional processing steps employed in the FISH protocol.

Uncertainty remains as to whether *T. whipplei* prefers intra- or extracellular growth environments. A detailed electron-microscopic study of intestinal WD [31] demonstrated that the majority of morphologically intact bacteria were located extracellularly in the lamina propria and that intracellular bacteria were in various stages of degradation. These findings are consistent with the results of more-recent work, which used FISH in intestinal biopsies [21] and which found *T. whipplei*–rRNA

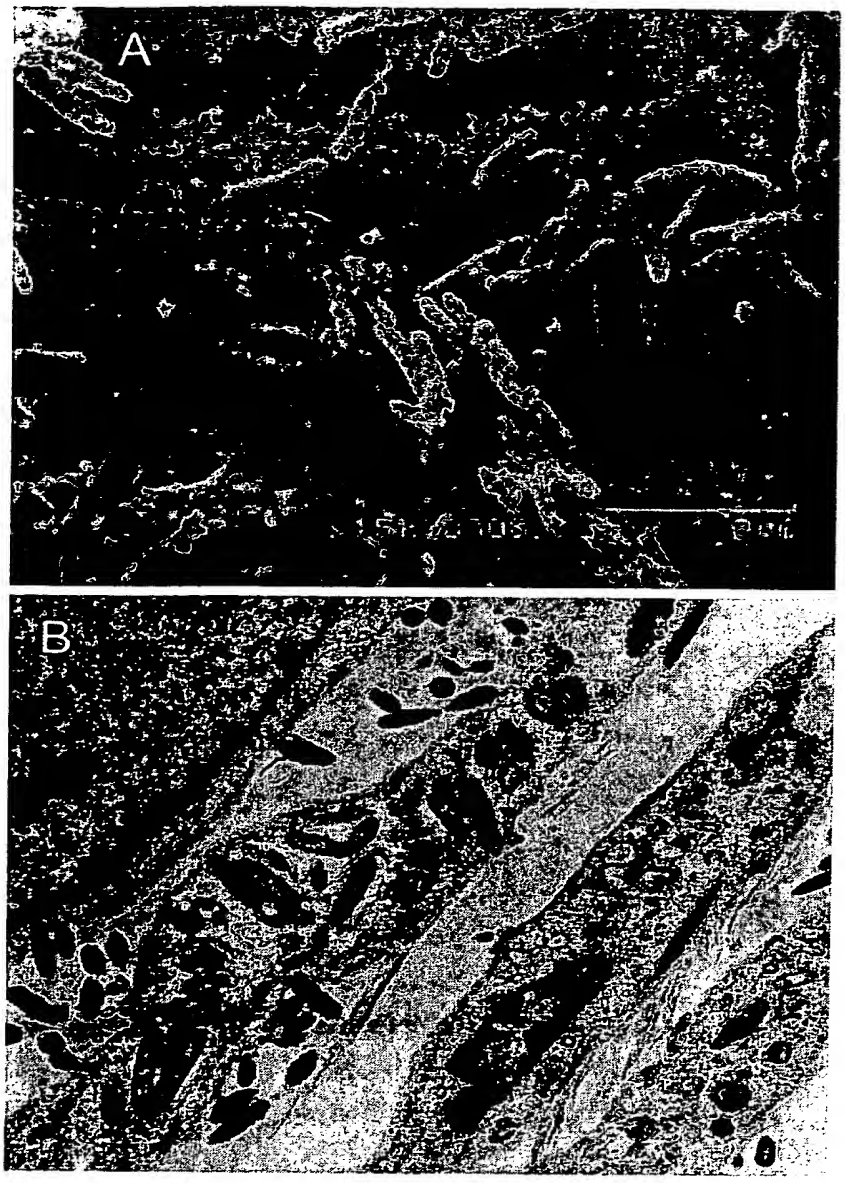

Figure 4. Electron micrographs of *Tropheryma whipplei* in fibroblast cell culture after the 14th passage. *A*, Results of scanning electron microscopy of strain TW08/27 (original magnification, ×20,000). *B*, Results of transmission electron microscopy of strain TW09/04 (original magnification, ×12,275).

hybridization signals, corresponding to metabolically active bacteria, in the lamina propria, directly subjacent to the epithelial basement membrane, but not inside cells. The location of the rRNA signal did not correspond to the inclusions characteristic of macrophages from patients with WD, inclusions that PAS shows to be positive for *T. whipplei*. On the other hand, Raoult et al. [10] reported intracellular growth in their fibroblast cell–culture system, which used PAS and immunofluorescence staining. In the present study, quantitative PCR with supernatant and with combined fractions indicated that *T. whipplei* grows in close association with fibroblasts but also grows in the cell-free supernatant. SEM clearly showed bacteria in extracellular locations (figure 4*A*); on the other hand, TEM showed intact bacteria in both intra- and extracellular locations (figure 4*B*). The host cells too appeared to be intact, and this obvious lack of cell damage is reminiscent of the paucity, in *T. whipplei* infection in humans, of both cell damage and inflammatory cellular infiltrate [16].

The *T. whipplei* isolate TW08/27 has been subjected to complete-genome sequencing [25] (see the http://www.sanger.ac.uk/Projects/T_whipplei/ Web site). Among fastidious and cultivation-resistant bacterial pathogens, the genome of *T. whipplei* is the third to be sequenced, after those of *Treponema pallidum* and *Mycobacterium leprae*. Although resistance to cultivation is uncommon among known pathogenic bacteria, the vast majority of bacteria in natural environments and in the commensal flora have not been cultivated in vitro [32, 33]. The extent to which currently uncharacterized or uncultivated bacteria might be involved in chronic idiopathic diseases is unclear [34]. In this context, *T. whipplei* is an attractive model organism with which to study such questions and, thus, to gather insight into related, important biological principles.

Acknowledgments

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Measles Virus Replication in Cells of Myelomonocytic Lineage Is Dependent on Cellular Differentiation Stage

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Measles virus (MV)-infected monocytes may have a central role in virus-induced immunosuppression. Our understanding of MV replication in monocytic cells is, however, incomplete. In this work we have investigated MV replication in cells of human myelomonocytic lineage with different maturation stages in order to study the effect of cellular maturation on virus infection. MV was able to infect human bone marrow myeloid granulocyte–macrophage colony-forming cells (CFC-GM) as well as monocytes and macrophages, but the replication cycle seemed to be regulated by the maturation stage of the cells. Virus infection in CFC-GM was productive, unlike in monocytes and macrophages, where an extensive viral RNA synthesis occurred and high amounts of proteins were synthesised without a remarkable release of infectious virus. Efficiency of viral macromolecular synthesis in macrophages was comparable to that of promonocytic cell line U-937 and human epithelial cell line A549, but in contrast to macrophages the cell lines highly supported productive infection. On the other hand, chemically induced maturation of the human promyelocytic and promonocytic cell lines HL-60, THP-1, and U-937 to more mature macrophage-like forms did not markedly alter the replication cycle of MV in these cell lines. Our results showed that MV replication in myelomonocytic cells varied depending on the maturation stage of the cells. The immature myelomonocytic cells supported productive virus infection, but the maturation process lead to cellular changes that caused a restriction of MV replication cycle partly at posttranscriptional and partly at posttranslational level. The metabolic milieu of monocytes and macrophages as such was sufficient to support extensive viral macromolecular synthesis.

Key Words: measles virus; myelomonocytic cells; bone marrow.

INTRODUCTION

Measles virus (MV), a single-stranded negative-sense RNA virus, is continuously globally an important pathogen causing 1 to 2 million annual deaths, mainly in developing countries. During acute illness, MV infects peripheral blood leukocytes (Berg and Rosenthal, 1961; Sullivan *et al.*, 1975) and monocytes are the major target cells (Salonen *et al.*, 1988; Esolen *et al.*, 1993). Infection causes strong immunosuppression with currently rather poorly known mechanisms. Infected monocytes may have a central role in the induction of immunosuppression. MV infection in monocytes changes many of their functions, e.g., by increasing production of interleukin-1β and reducing levels of tumour necrosis factor-α and interleukin-12 (Leopardi *et al.*, 1992; Ward *et al.*, 1991; Karp *et al.*, 1996). The expression of MHC class II molecules and the antigen-presenting function of HLA-DR, -DQ, and -DP molecules are enhanced in MV-infected monocytes *in vitro*, but no change is detected in infected promonocytic cell line THP-1, indicating that activation and/or maturation stage of the cells may play a pivotal role in virus-induced events (Leopardi *et al.*, 1993).

Besides disturbances caused by MV infection in immunological functions, infected cells of immune system can transport the virus to various target organs. MV replication in primary monocytes is highly restricted (Vainionpää *et al.*, 1991; Karp *et al.*, 1996), whereas immature cord blood monocytes from neonates support productive virus infection (Sullivan *et al.*, 1975). Moreover, the report by Bashle and co-workers (1985) suggests that MV replication may vary with the stage of differentiation or maturation of the cells. They have shown MV antigens in osteoclasts from patients with Paget's disease. Osteoclasts originate from bone marrow via mononuclear cells and have characteristics similar to macrophages. MV antigens were detected in both nuclei and cytoplasms of osteoclasts. This kind of intracellular distribution of MV antigens is known to be typical for persistently MV-infected cells (Norrby *et al.*, 1982; Chui *et al.*, 1986). Although an increasing number of studies indicate an important role of infected monocytes in MV immunopathogenesis, our knowledge about virus replication in myelomonocytic cells is limited. It is therefore of interest to analyse MV infection in more details in myelomonocytic cells with different maturation stages

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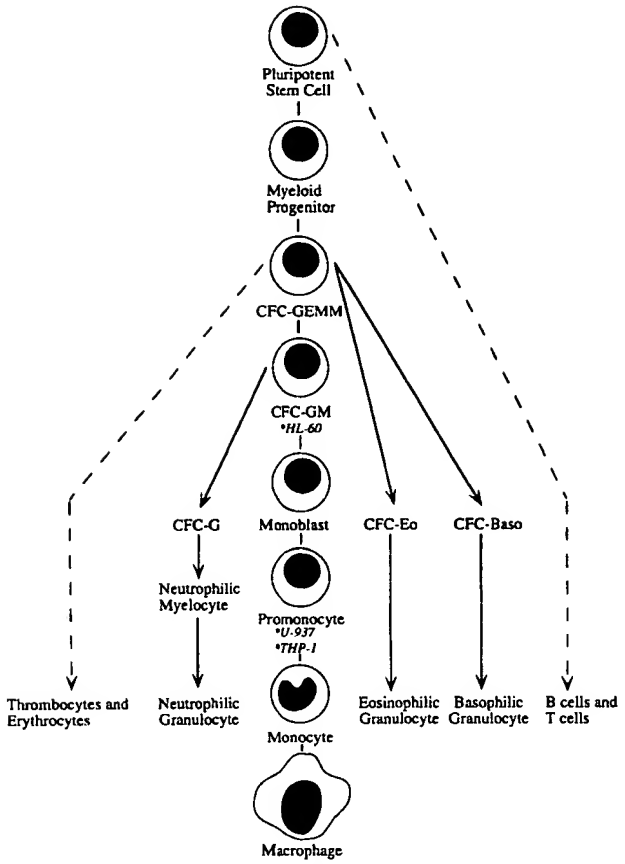

FIG. 1. Maturation and differentiation of myelomonocytic cells.

and study if host cell factors induced during maturation can modulate virus replication cycle.

To understand better MV infection in monocytic cells we have analysed MV replication in myelomonocytic cells with different maturation stages, from human bone marrow granulocyte/macrophage progenitors to monocytes and macrophages, as well as in promyelocytic and promonocytic cell lines HL-60, THP-1, and U-937. MV replication was productive in immature cells and restricted in mature monocytes/macrophages and the restriction occurred both at posttranscriptional and at posttranslational levels.

RESULTS

Maturation and differentiation of the cells of myelomonocytic lineage and the maturation stages of the promonocytic and promyelocytic cell lines THP-1, U-937, and HL-60 used in this study are shown in Fig. 1.

Myelomonocytic progenitors support productive MV replication

The mononuclear bone marrow cells divide approximately six times during the culturing period of 14 days, and granulocyte–macrophage colony-stimulating factor (GM-CSF) present in the culture medium supports exclusively the growth of only granulocyte–macrophage colony-forming cells (CFC-GM). Therefore, each colony is a product of one single progenitor cell and contains a mixture of original-type progenitor cells and more or less differentiated but still very premature cells of the myeloid lineage.

In contrast to mature monocytes, MV was able to replicate productively in progenitor cells of myelomonocytic lineage. The amount of infectious virus in culture medium increased from 8×10^1 PFU/ml (representing the rest of inoculum virus in medium after washings) to 3×10^3 PFU/ml (3 days p.i.) being at the same level still at day 5 p.i. Figure 2 shows a one-step growth curve of MV in CFC-GM. Immunofluorescent staining of the colonies showed sporadic MV antigen-positive cells, suggesting that infection was limited to a certain cell type (data not shown).

MV replication in monocytes/macrophages is restricted

We have earlier shown that MV replication in monocytes is incomplete. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) and Ca^{2+}-ionophore, which enhance protein kinase C activity and change a nonproductive MV replication to a productive one in peripheral blood mononuclear cells (PBMC), could not induce production of infectious virus in monocytes (Vainionpää *et al.*, 1991). In this study we examined whether differentiation of monocytes by IL-3 and/or GM-CSF would increase MV infection in monocytes/macrophages. For these experiments, monocytes were infected with MV, washed, and incubated for 3 days, after which maintenance medium was replaced by medium containing various concentrations of IL-3 (1, 10, or 25 IU/ml) and/or GM-CSF (10, 25, or 50 IU/ml). Incubation was continued for further 3 days and the specimens were collected for infectivity titration assay. Although more than 70% of monocytes in cultures contained intracellular MV proteins when examined by im-

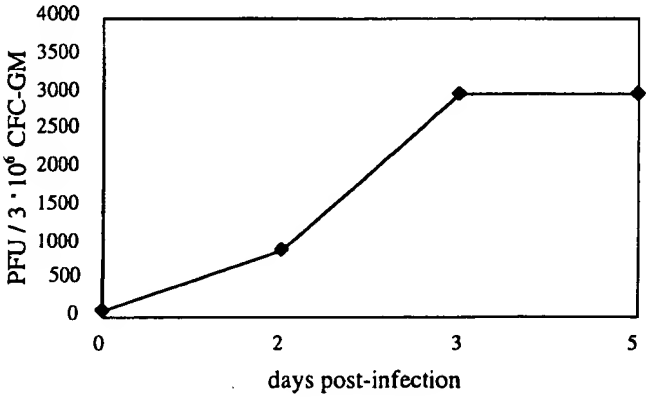

FIG. 2. One-step growth curve of measles virus in granulocyte–macrophage colony-forming cells (CFC-GM) quantified from the culture medium by plaque titration assay in Vero cells.

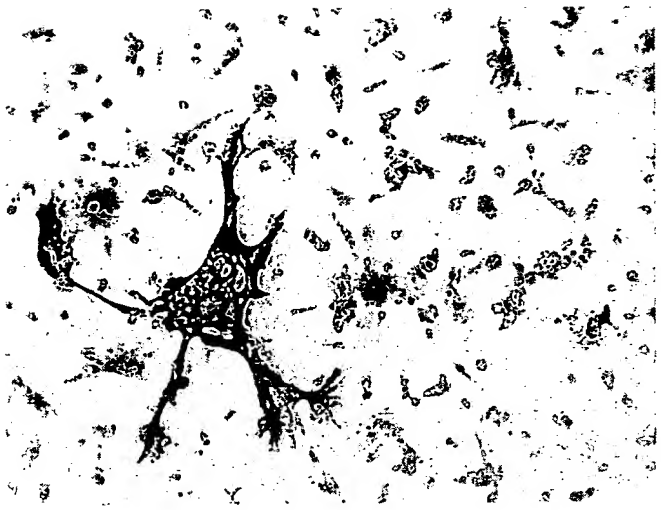

FIG. 3. MV infection of macrophages. Monocytes were infected with MV and then incubated in mitogen-stimulated medium for 3 days, after which the cells were fixed with 75% acetone and stained with the rabbit anti-measles virus serum and the HRP-conjugated goat anti-rabbit IgG. The figure shows one MV-infected, multinucleated macrophage and several smaller uninfected and infected mononucleated macrophages.

munofluorescent staining, neither the individual cytokines IL-3 or GM-CSF nor their mixture were able to increase MV replication (data not shown).

In order to further study the effect of maturation of monocytes to macrophages on their capacity to support MV replication, monocytes were infected, incubated for 3 days, and the maintenance medium was replaced by "mitogen-stimulated" medium or by medium from unstimulated PBMC culture. After 3 days the cells treated with mitogen-stimulated medium appeared as macrophage-like cells, and most of them were strongly positive for MV antigens when stained by the rabbit anti-measles virus antibodies. The cultures also contained MV antigen-positive multinucleated giant cells, one of which has been shown in Fig. 3. Maturation of infected monocytes to macrophages did not, however, change a restricted virus replication to a productive stage. Freezing and thawing did not have any effect on the release of infectious virus from macrophages either.

MV replication in monocyte-derived macrophage cultures was analysed in more detail by Northern and Western blotting techniques as well as by the infectivity titration assay. MV replication in the promonocytic cell line U-937 and in the epithelial cell line A549 was analysed for comparison. The macrophage cultures were maturated in macrophage SFM medium for 14 days as described under Materials and Methods. Figure 4 shows that virus RNA synthesis was more effective in macrophage cultures than the RNA synthesis in U-937 cells and comparable to the RNA synthesis in A549 cells, which are known to productively support MV replication. Viral proteins in macrophages were detected as well, indicating ongoing virus protein synthesis, although the protein amount was much lower than the proteins in the cell lines (Fig. 5). No release of infectious virus from macrophage cultures occurred, however. In contrast, virus replication in U-937 cells as well as in A549 cells was highly productive, the virus titres being 2×10^5 PFU/ml and 7×10^4 PFU/ml, respectively. These results showed that the metabolic milieu of monocytes/macrophages support MV macromolecule synthesis and the inhibition of effective virus production occurs at both posttranscriptional and posttranslational levels.

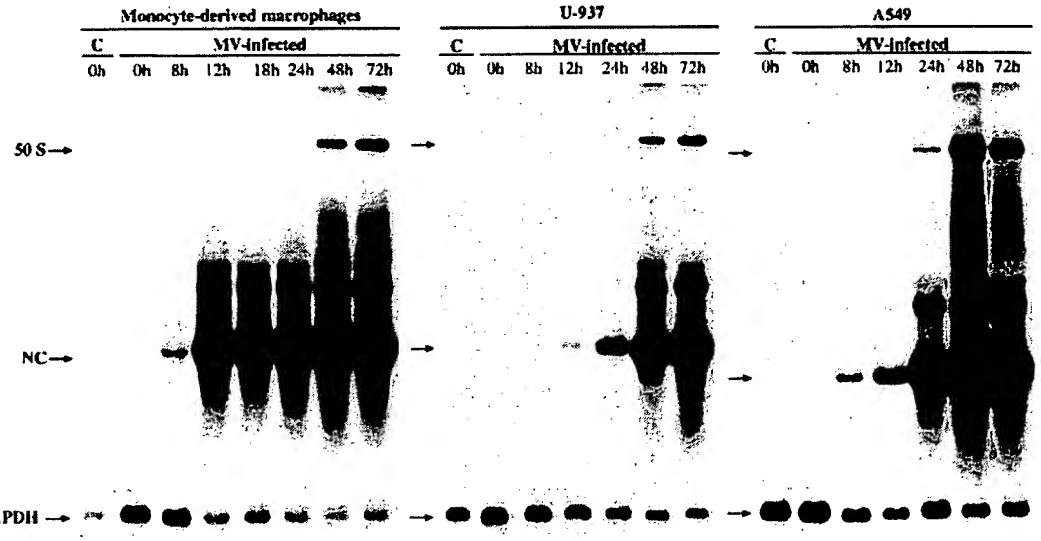

FIG. 4. Northern blotting analysis of total RNA in MV-infected monocyte-derived macrophages, U-937, and A549 cells. The specimens were collected at the times indicated. The filters were hybridised with the MV NC cDNA probe as well as with the GAPDH cDNA, which was used as an internal standard. The positions of the MV genomic size RNA 50S and NC are marked by the arrows. C, total RNA from uninfected cells.

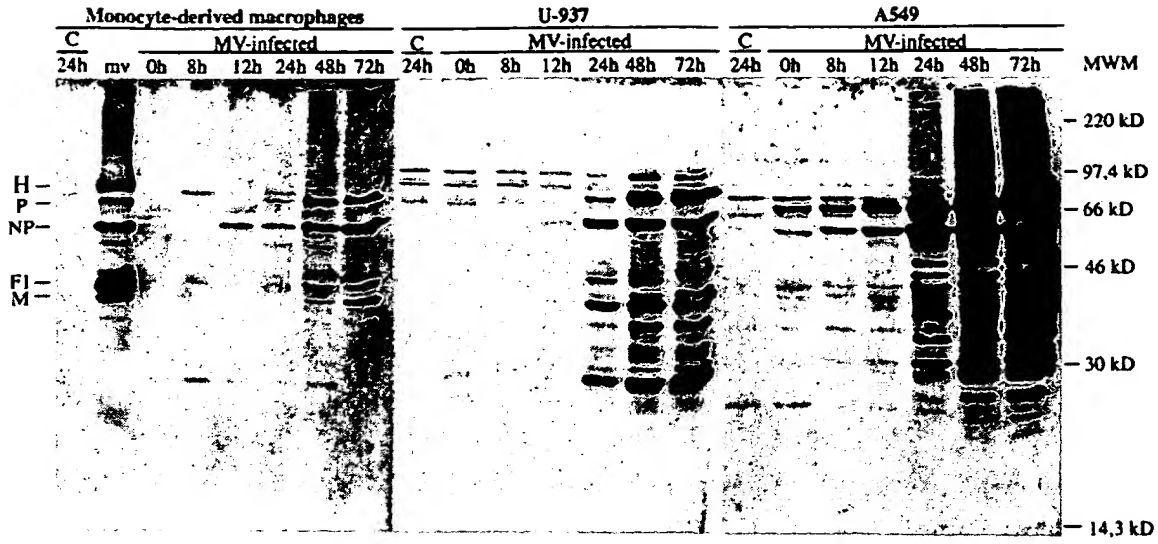

FIG. 5. Western immunoblot detection of MV proteins in monocyte-derived macrophages, U-937, and A549 cells. The specimens were collected at the times indicated, separated by SDS–PAGE, blotted onto nitrocellulose membrane, incubated with the rabbit anti-measles virus serum and the anti-rabbit HRP-conjugate, and detected by enhanced chemiluminescence. The positions of MV structural proteins are indicated at the left and the molecular weight markers at the right. mv, inoculum virus. C, uninfected cells.

MV infection in promyelocytic and promonocytic cell lines

For comparison, we studied MV replication also in the human promyelocytic and promonocytic cell lines HL-60, THP-1, and U-937. These cell lines, each of which represents a different stage of maturation, are widely used as *in vitro* models to study interactions between viruses and monocytes because they display a number of monocytic characteristics. They can also be induced to mature with phorbol esters such as TPA to more mature macrophage-like cells. HL-60 cells are closely related to early progenitor cells. They have bilinear differentiation potential and they can be chemically induced to differentiate to either granulocytic or monocytic cells. THP-1 and U-937 cell lines are from the monocyte/macrophage lineage and U-937 cells represent a later maturation stage. Chemically induced maturation has been reported to change the permissivity of these cell lines for replication of many other viruses (Roivainen and Hovi, 1989; Tenney and Morahan, 1991; Weinshenker *et al.*, 1988).

All three cell lines supported viral RNA synthesis to a similar extent (Fig. 6) when followed by spot hybridisation and measuring the radioactivity of the spots by liquid scintillation counting. However, the amount of released infectious virus did not correlate with the amount of viral RNA. The virus release was most effective in the promonocytic cell lines THP-1 (1×10^5 $TCID_{50}$) and U-937 (1×10^5 $TCID_{50}$). In contrast, the promyelocytic cell line HL-60 supported virus release only at a minimal level (5×10^1 $TCID_{50}$).

Because MV replication in PBMC is dependent on cellular activation stage (Lucas *et al.*, 1978; Hyypiä *et al.*, 1985), we wanted to compare the proliferation capacity of these cell lines by measuring their DNA synthesis by [^{3}H]thymidine incorporation. As shown in Fig. 7, THP-1 and U-937 cells were metabolically more active than HL-60 cells, and ongoing MV replication in these cells caused strong suppression of cellular DNA synthesis which was most evident in U-937 cells.

TPA is known to cause maturation of HL-60, U-937, and THP-1 cells (Tsuchiya *et al.*, 1982). In order to study the effect of maturation on MV replication, the cell lines were

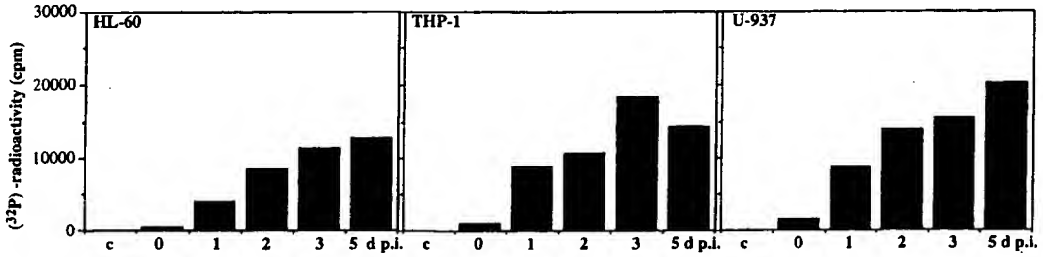

FIG. 6. MV-specific RNA synthesis in the promyelocytic and promonocytic cell lines HL-60, THP-1, and U-937 analysed by spot hybridisation and by measuring the radioactivity of the spots by liquid scintillation counting.

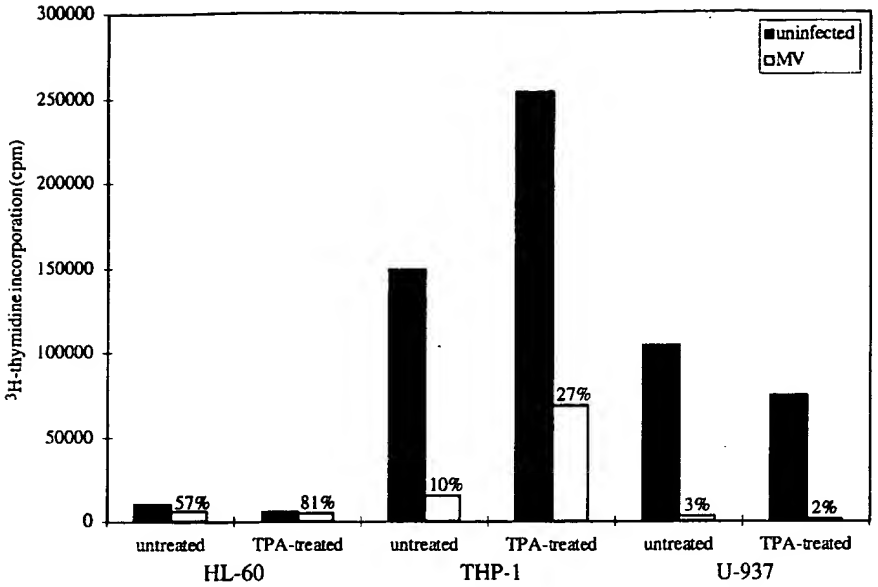

FIG. 7. DNA synthesis, as measured by [^{3}H]thymidine incorporation, in MV-infected and uninfected monocytic cell lines, which were either untreated or pretreated with TPA (1.0 nM) for 24 h before infection. The mean cpm (counts per minute) values of three parallel cultures are shown. The cpm values of MV-infected samples relative to corresponding uninfected samples are presented in percentages.

treated with 1 nM of TPA for 24 h before infection. The changes caused by maturation on cell proliferation were followed by measuring DNA synthesis by [^{3}H]thymidine incorporation (Fig. 7). TPA stimulated the proliferation of THP-1 cells, but caused suppression of DNA synthesis in HL-60 and U-937 cells. No clear-cut effect of cellular maturation on virus RNA synthesis as determined by spot hybridisation or on virus production was detected (data not shown). Our results showed that although TPA-induced maturation caused changes in cellular activity, no similar decrease/increase was observed in virus replication, indicating that MV replication did not depend entirely on the metabolic activity of the host cell.

DISCUSSION

Our results showed that human bone marrow progenitor cells were susceptible to productive MV replication. During the maturation of myelomonocytic cells some changes occurred, which lead to restriction of MV infection. Monocytes/macrophages supported extensive viral RNA and protein synthesis, but no clear-cut release of infectious virus was observed.

Infected monocytes may have a central role as mediators of immunosuppression, which is most probably a multifactorial event. On one hand, active virus replication is needed for suppression, because UV-inactivated virus is able to suppress cell proliferation in a much less extent than infectious virus does. On the other hand, interaction of MV glycoproteins with the surface of uninfected PBMC is sufficient to induce immunosuppression (Schlender *et al.*, 1996). Evidently cell cycle arrest of infected B- and T-lymphocytes is also partly responsible for suppression (McChesney *et al.*, 1987, 1988). MV is known to cause many cytokine dysfunctions of monocytes (Griffin and Ward, 1993), e.g., down-regulation of IL-12 (Karp *et al.*, 1996), which is known to be critical for the generation of cell-mediated immunity. This down-regulation was induced also by UV-inactivated virus. One obvious mechanism involved in immunosuppression is apoptosis observed in monocytes, dendritic cells, and T-lymphocytes in MV-infected cultures. MV-infected dendritic cells have been shown to induce apoptosis also in uninfected T-lymphocytes (Fugier-Vivier *et al.*, 1997).

As described above, an increasing amount of information suggests the central role of monocytic cells in MV immunopathogenesis, but much less is known about virus replication in myelomonocytic cells. Sullivan *et al.* (1975) have reported that the immature cord blood monocytes from neonates support a complete replication cycle of MV, and because a considerable proportion of monocytes in circulation can be immature, MV infection in these cells can contribute to the spreading of infection. An interesting question is whether MV can infect bone marrow progenitor cells. MV is known to cause life-long immunity, and it has been proposed that the persistence of the virus in bone marrow could be a reason for prolonged antibody production after primary infection. In this work we demonstrated that MV was able to infect bone marrow myeloid progenitor cells *in vitro*, and CFC-GM supported productive MV infection. Bone marrow cells are known to be affected in a number of different virus infections. For instance, human parvovirus B19 can cause aplastic crisis in patients with sickle cell anaemia (Pattison *et al.*, 1981), cytomegalovirus in-

fects both stromal and hematopoietic progenitor cells (Maciejewski *et al.*, 1992), and human herpes virus 6 (Knox and Carrigan, 1992) and dengue virus (Nakao *et al.*, 1989) infect bone marrow cells. To our knowledge this is the first time when MV has been shown to infect and replicate in human bone marrow progenitor cells.

There is variation in reports concerning MV replication in monocytes. Joseph *et al.* (1975) have described productive MV replication in monocytes, whereas we and others have shown that in mature monocytes/macrophages MV replication is highly restricted, and stimulation with various extracellular mitogens, which activate different biochemical pathways, does not activate the silent infection to a productive one (Vainionpää *et al.*, 1991; Karp *et al.*, 1996). This variation might be caused by different factors, e.g., maturation stage of monocytes. Also the susceptibility of monocytes from different individuals can vary.

In this report we have shown that the metabolic milieu of monocytes/macrophages as such could support MV macromolecular synthesis, because extensive viral RNA and protein synthesis occurred. The active virus protein synthesis, without release of infectious virus, in monocytes/macrophages may lead to accumulation of virus glycoproteins on cell surface, and this phenomenon could be an important factor in monocyte-mediated immunosuppressive events. Also the report by Bashle and co-workers (1985), describing persistent-type MV infection in osteoclasts, suggests restriction of MV replication in mature cells of the same myelomonocytic lineage. Cirino and co-workers (1993) have reported that respiratory syncytial virus (RSV), a member in the family of Paramyxoviridae, replicates productively in freshly isolated alveolar macrophages, but *in vitro* differentiation of monocytes into macrophages results in a significant, time-dependent decrease in production of infectious virus. The mechanisms by which cellular differentiation restricts MV and RSV replication are still unknown.

As a conclusion, our results suggest that the maturation stage of myelomonocytic cells may have an important role in the pathogenesis of measles. On one hand, immature monocytic cells can support productive virus replication, which can lead to the dissemination of the virus in the body. On the other hand, mature monocytes/macrophages support extensive virus RNA and protein synthesis, without release of infectious virus. These cells containing high amounts of virus proteins can be responsible for other dysfunctions, such as immunosuppression occurring in MV pathogenesis.

MATERIALS AND METHODS

Virus

A wild-type measles virus (Halonen-strain, Vainionpää *et al.*, 1978) with a high infectivity titre ($>1 \times 10^7$ PFU/ml) was used throughout the study. The inoculum virus was propagated in Vero cells. For RNA and protein isolation the cells were infected at a multiplicity of infection (m.o.i.) of 5. In all the other works cells were infected at a m.o.i. of 1. After the adsorption time of 60 min those cultures later checked for virus production were thoroughly washed, and fresh maintenance medium was added.

Bone marrow progenitor cells

CFC-GM cultures were prepared as described earlier by Vuorinen *et al.* (1996). Briefly, mononuclear cells were obtained by the COBE 2991 Model I blood cell processor (COBE) from Ficoll–Paque (Pharmacia, Uppsala, Sweden)-separated heparinised bone marrow collected from autologous transplantation patients (disease-free at the time of the collection). The *in vitro* colonies of hematopoietic progenitors were cultured by the methyl cellulose technique originally developed by Pike and Robinson (1970) with the modification of Guilbert and Iscove (1976). Mononuclear bone marrow cells (2×10^5/ml) were mixed with culture medium containing 1% methyl cellulose, 20% foetal bovine serum (FBS, HyClone), 1% delipidated and deionised bovine serum albumin (Sigma, Cell Culture), 1×10^{-4} M β-mercaptoethanol, and 0.5 mg/ml fully iron-saturated human transferrin (Behringwerke) in Iscove's Modified Dulbecco's minimum essential medium (Gibco) and added to plastic Petri dishes. CFC-GM were cultured in the presence of GM-CSF (Leucomax, Sandoz, Schering-Plough), which supports the growth of only granulocytic and monocytic lineage cells. The plates were incubated for 14 days at 37°C in a fully humidified atmosphere with 5% CO_2.

The propagation of the bone marrow cultures was performed by Central Laboratory, Department of Haematology, Turku University Central Hospital to check the viability of autologous bone marrow transplantation material. The propagation took 2 weeks after which this anonymous waste material could be used for our purpose.

For virus infection the monocytoid colonies were picked under a light microscope, infected with MV (1 m.o.i.), washed, and suspended into the culture medium (without methyl cellulose) as described above.

Other cells and cell lines

Human peripheral blood puffy coat fractions of healthy blood donors were obtained from The Finnish Red Cross Blood Transfusion Service, Turku. To isolate mononuclear cells, the puffy coat cells were centrifuged at 1600 rpm for 45 min through a Ficoll–Paque cushion. The monocytes were enriched by adherence to polystyrene plastic plates, and nonadherent cells were removed by washing with Hanks' balanced salt solution. The cell preparations contained more than 90% monocytes as estimated by their light scattering properties in fluorescence-activated cell sorter analysis. The cells were

maintained at 37°C with 5% CO_2 in RPMI 1640 medium (Gibco) supplemented with 10% human AB serum; in MV-infected cultures 10% FBS was used instead of human serum.

Monocyte-derived macrophages were cultured from monocytes (isolated as described above) in macrophage SFM medium (Gibco) containing 10 ng/ml of GM-CSF for 14 days, during which period the medium was replaced every second day by fresh medium. Maturation of monocytes to macrophages was also done by incubating the MV-infected monocytes in mitogen-stimulated medium, which was a supernatant from concanavalin A (Con A)-treated uninfected PBMC. For preparation of such a supernatant, uninfected PBMC were treated with Con A (10 μg/ml) overnight, after which Con A was washed out with medium containing 0.3 M α-methyl-D-mannoside. Incubation was continued for 2 days, and the cells were pelleted and the supernatant was used as mitogen-stimulated medium. Maturation of the monocytes was detected based on morphology.

The human promyelocytic cell line HL-60 (ATCC CCL-240) and the promonocytic cell lines THP-1 (ATCC TIB-202) and U-937 (ATCC CRL-1593) were cultured in RPMI 1640 medium supplemented with 10% FBS. The maturation of the cell lines was induced by treatment with 1.0 nM of TPA (Sigma, St. Louis, MO) overnight. The human epithelial cell line A549 (ATCC CCC-185) was maintained in Ham's F-12 medium.

Assays for virus infectivity

The amount of infectious virus was determined by incubation of serial 10-fold dilutions of supernatants from infected cells on confluent monolayers of Vero cells. Each dilution was assayed in duplicate. After 5 to 7 days, the virus titre was determined after either a standard plaque counting (Vainionpää *et al.*, 1978) or a reading of the $TCID_{50}$ by light microscopy.

Immunostaining of cells

Immunoperoxidase staining of macrophages was done as described by Waris *et al.* (1990). Briefly, the cells were fixed on polystyrene plastic plates with cold 75% acetone and incubated first with the rabbit anti-measles virus serum and then with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories; diluted 1/400). 3-Amino-9-ethylcarbazole dissolved in dimethylformamide was used as chromogen.

Nucleic acid hybridisation

A cDNA clone composed of 1.6 kb of MV nucleoprotein mRNA in a pBR322 vector was used as a probe. Labelling of the probe, treatment of the cells, and hybridisation procedures were done as described by Hyypiä *et al.* (1985).

RNA isolation and Northern blotting

For RNA analysis, the specimens (10×10^6 cells) were harvested at indicated times. Total cellular RNA was isolated by guanidium isothiocyanate lysis followed by CsCl centrifugation 35,000 rpm overnight at 15°C (Chirgvin *et al.*, 1979). Equal amounts of RNA (20 μg) were electrophoresed in a 0.8% formaldehyde–agarose gel, transferred to a Zeta-Probe GT genomic tested blotting membrane (Bio-Rad Laboratories, CA) and hybridised with MV nucleocapsid-specific cDNA probe (a gift of Dr. T. Wong, University of Washington, Seattle, WA), labelled with [α-^{32}P]dCTP (Amersham) by a random primed DNA labelling kit. Determination of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by a cDNA probe (Fort *et al.*, 1985) was used as an internal control.

Immunoblotting

For virus protein analysis, the cells were lysed in RIPA buffer (50 mM Tris–HCl buffer, pH 8.0, containing 1% NP-40, 0.4% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin and aprotinin) and centrifuged 14,000 rpm for 30 min to remove insoluble material. Protein concentrations were estimated by Bio-Rad protein assay kit. The proteins (100 μg/lane) were separated by 10% SDS–PAGE, transferred electrically by Semi Dry Blot Pegasus (PHASE GmbH, Germany) onto Schleicher & Schuell nitrocellulose filter. After the blocking with 5% milk, the filters were incubated with the rabbit anti-measles virus antibodies (dilution 1:125 in phosphate-buffered saline containing 1% Triton X-100 and 5% milk powder) for 3 h at 37°C and then with the anti-rabbit HRP-conjugate. The bands were visualised by ECL chemiluminescence system (Amersham).

Cell proliferation assay

DNA synthesis of the untreated and TPA-maturated cell lines was measured by [^{3}H]thymidine incorporation. For maturation, the cells were treated with TPA at a concentration of 1 nM for 24 h before the infection and cultivated at a density of 1×10^5 cells/200 μl. [^{3}H]thymidine (Amersham; 0.5 μCi per well) was added 20 h before harvesting. The cells were harvested 4 days postinfection with a multichannel semiautomatic cell harvester (Skatron), and the radioactivity was measured in a 1217 Rackbeta liquid scintillation counter (Wallac, LKB).

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Growth pattern of the human promyelocytic leukaemia cell line HL60. Foa P, Maiolo AT, Lombardi L, Toivonen H, Rytomaa T, Polli EE. Cell Tissue Kinet. 1982 Jul;15(4):399-404

In order to characterize the growth pattern of the human promyelocytic leukaemia cell line HL60, its kinetic parameters were studied. The doubling time was calculated from serial cell counts, the duration of the various cell cycle phases from the analysis of the labelled mitoses curve, and quiescent population from continuous labelling experiments. Proliferation in culture was exponential up to a saturation density of about 3.0 x 10(6) cells/ml, with a doubling time of 34.0 hr. The cell cycle duration was 24.3 +/- 4.1 hr (SD), and that of the cell cycle phases was: G1, 3.8 +/- 2.2 hr; S, 15.1 +/- 3 hr; and G2, 4 +/0 1.2 hr. The growth fraction was 0.85, and cell loss was restricted to the quiescent cells. The HL60 cell line, with fully characterized kinetics, provides a useful tool for the vitro study of substances which may affect human leukaemic myelopoietic proliferation.

Articles

Sequencing and analysis of the genome of the Whipple's disease bacterium *Tropheryma whipplei*

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Summary

Background Whipple's disease is a rare multisystem chronic infection, involving the intestinal tract as well as various other organs. The causative agent, *Tropheryma whipplei*, is a Gram-positive bacterium about which little is known. Our aim was to investigate the biology of this organism by generating and analysing the complete DNA sequence of its genome.

Methods We isolated and propagated *T whipplei* strain TW08/27 from the cerebrospinal fluid of a patient diagnosed with Whipple's disease. We generated the complete sequence of the genome by the whole genome shotgun method, and analysed it with a combination of automatic and manual bioinformatic techniques.

Findings Sequencing revealed a condensed 925 938 bp genome with a lack of key biosynthetic pathways and a reduced capacity for energy metabolism. A family of large surface proteins was identified, some associated with large amounts of non-coding repetitive DNA, and an unexpected degree of sequence variation.

Interpretation The genome reduction and lack of metabolic capabilities point to a host-restricted lifestyle for the organism. The sequence variation indicates both known and novel mechanisms for the elaboration and variation of surface structures, and suggests that immune evasion and host interaction play an important part in the lifestyle of this persistent bacterial pathogen.

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Introduction

First described in 1907, Whipple's disease is a multisystem disorder, involving the intestinal tract and various other organs.[1] The disease is fatal if left untreated. The clinical presentation is heterogeneous. Frequently, patients complain of arthralgias, chronic diarrhoea, and weight loss, and less often from central nervous or cardiac manifestations, for years before diagnosis. Because of its varied manifestations, the disease has found its way into the differential diagnosis of many clinical disorders.

Since the 1960s, electron microscopy studies have consistently shown small, uniform, rod-shaped bacteria in affected tissues, measuring about 0·2×1·5–2·5 μm.[2] The bacterial cell wall has a trilaminar appearance, with an outer membrane that is proposed to be of host origin. Many attempts were undertaken to propagate this bacterium in the laboratory, but it proved resistant over many decades to cultivation. Broad-range bacterial 16S rDNA PCR followed by phylogenetic analysis[3,4] has placed the bacterium within the Gram-positive bacteria with high G+C content (class actinobacteria). The bacterium holds an intermediate position between actinobacteria with the common group A and the uncommon group B peptidoglycan, and is not closely related (16S rDNA divergence >7%) to any cultivated representative.[3] Isolation of the bacterium *Tropheryma whipplei* was achieved in 2000, in a long-term culture system with human fibroblasts, with a reported generation time of 18 days.[5]

Many aspects of Whipple's disease and *T whipplei* remain poorly understood, including clinical, histological, and epidemiological features of the illness, and metabolic capabilities, ecology, and interactions of the bacterium with the human host. Among the observations and unproven propositions are a close association of this bacterium with human beings, a possible bacterial environmental niche,[2] and a predilection for causing disease in outdoor workers.[1] Some have proposed that patients with Whipple's disease have subtle immune defects.[6] Additionally, the bacterium has a unique cell wall, it localises in the intestinal lamina propria, and it elicits a cellular response that is composed almost entirely of macrophages, with accumulation of bacterial cell-wall remnants in these cells. It has been fairly resistant to cultivation in vitro, and seems to depend on human cell-associated factors for growth, with an in-vitro doubling time that is among the longest known for bacteria.

Genome sequences have provided many insights and clues about bacterial functional capabilities and evolution. By contrast with the medically important bacteria, nearly all bacteria in natural environments and most in the commensal flora have not been cultivated in vitro.[7] The genomes of two other cultivation-resistant human pathogens, *Treponema pallidum*, the agent of syphilis, and *Mycobacterium leprae*, the agent of leprosy, have revealed features that are uncommon among other bacteria with

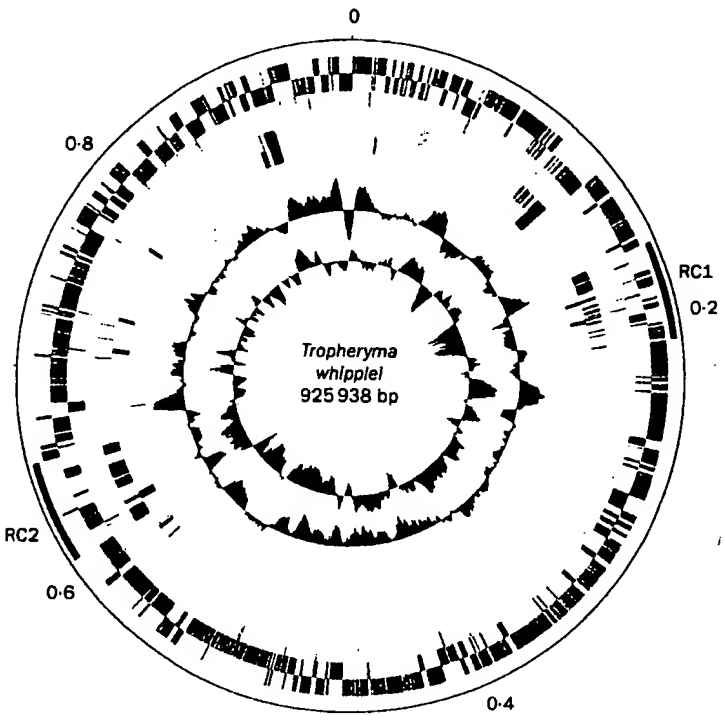

Figure 1: **Circular representation of the *T whipplei* chromosome**
The outer scale shows the size in Mb. RC refers to repeat clusters (see text). From the outside in, circles 1 and 2 show all genes transcribed clockwise and anticlockwise (see colour code below); circle 3 shows genes for stable RNA (blue); circle 4 shows DNA repeat regions (pale blue=coding repeats, orange=non-coding repeats); circle 5 shows the members of the WISP family (green); circle 6 shows the positions of base-pair sequence variations; circle 7 shows a plot of G+C content (in a 10 Kb window); circle 8 shows a plot of GC skew ([G-C]/[G+C]; in a 10 Kb window). Colour coding for genes: dark blue=pathogenicity or adaptation, black=energy metabolism, red=information transfer, dark green=membranes or surface structures, cyan=degradation of macromolecules, purple=degradation of small molecules, yellow=central or intermediary metabolism, light blue=regulators, orange=conserved hypothetical, pale green=unknown, and brown=pseudogenes.

sequenced genomes. For example, *T pallidum*, with a 1·14 Mb genome, is deficient in genes for catabolic and biosynthetic pathways,[10] and *M leprae* (which contains numerous pseudogenes and evidence of substantial decay and reductive evolution) has maintained almost all biosynthetic pathways while substantially reducing its catabolic and energy-production pathways, leading to its obligate intracellular lifestyle in people.[11]

Until now, genomic characterisation of *T whipplei* has been restricted to the DNA sequences for the rRNA operon and two housekeeping proteins (RpoB and GroEL). The study of the *T whipplei* genome provides an opportunity for new insight into the biology of this enigmatic pathogen and its interaction with human beings, the development of new diagnostic and preventive strategies, as well as the fundamental principles governing the evolution of host-adapted microorganisms. Our aim was, therefore, to generate and analyse the sequence of the *T whipplei* genome.

Methods

We isolated the *T whipplei* strain TW08/27 from the cerebrospinal fluid of a woman in Germany 2 years after presentation with severe weight loss. Her diagnosis of Whipple's disease was based on intestinal histology and 16S rDNA PCR at the time of presentation. The patient had received an initial 2-week course of treatment with penicillin plus streptomycin, followed by 1 year of cotrimoxazole, and had had a therapy-free interval of 1 year before the sample was taken.

Culture was done with a previously described human fibroblast system[12] initially on MRC-5 cells (CCL-171; American Type Culture Collection, Manassas, VA, USA) and then, in parallel, beginning with the 13th passage, on primary human foreskin fibroblasts (gift from E Mocarski, Stanford University, CA, USA). The initial inoculum consisted of about 10^3–10^4 bacteria in 0·5 mL cerebrospinal fluid. We detected growth after 6 weeks, as assessed by qualitative PCR for *T whipplei*,[13] by quantitative PCR, and by documentation of bacteria by using YO-PRO-1 nucleic acids stain (Molecular Probes, Eugene, OR, USA). 15 culture passages were completed over 17 months, by inoculating 1/4 to 1/5 volume of culture supernatant with bacteria onto fresh fibroblast monolayers. We measured bacterial growth by quantitative competitive PCR with *T whipplei*-specific primers[13] and a synthetic internal standard molecule, and estimated a generation time of 4 days. We identified bacteria in the culture by fluorescence in-situ hybridisation with a *T whipplei*-specific probe and a bacterial broad-range probe,[13] and by broad-range PCR, spanning 1443 bp of 16S rDNA. All bacterial cells hybridised with the *T whipplei* probe, indicating the absence of contaminants. 16S rDNA, amplified with broad range primers after the 15th passage, had two mismatches with the sequence of *T whipplei* (EMBL accession number: X99636) at unpaired positions. We harvested about 10^9–10^{10} bacteria from the culture supernatant of 50, 150 cm^2 tissue-culture flasks by differential centrifugation (10 min at 950 *g* to remove cell debris, then 20 min at 10 000 *g* to pellet bacteria), and extracted genomic DNA from the bacterial pellet with lysozyme and proteinase K digestion, phenol/chloroform extraction, and isopropanol precipitation.

We sheared chromosomal DNA by sonication, and size fractionated it on agarose gels, before cloning into pUC19 and pMAQ1b vectors (http://www.sanger.ac.uk/Teams/Team53/psub/ref.shtml). End sequences from these clones were generated with dye terminator chemistry on ABI3700 automated sequencers. The final sequence was obtained

Panel 1: General features of the *T whipplei* genome

Size	925 938 bp
G+C content	46·3%
Coding sequences	764*
Coding content	84·4%
Average gene length	998 bp
rRNA	1 (16S–23S-5S)
tRNA	41
Other stable RNAs	1

*of which one is a pseudogene

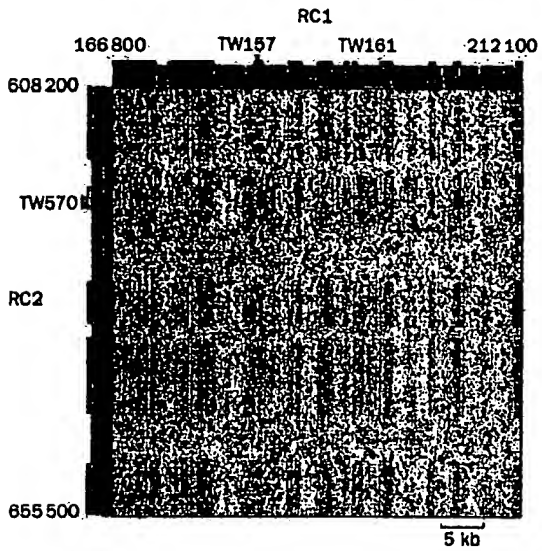

Figure 2: **Dotplot comparison of RC1 and RC2**
The centre of the figure shows the DNA:DNA similarities between RC1 and RC2, with similar sequences represented by dots or lines. The degenerate repetitive structure of the non-coding regions can be seen, along with the copies of these repeats in the WiSP proteins. The conserved N-terminus of the WiSP protein is also visible. The boxes with arrows around the plot show the genes encoded by the region, with the colour code as in figure 1. The non-coding repeat regions are shown by the outermost orange boxes, with the similar repeats in TW157 and TW570 indicated by hatched orange boxes. The positions of variable sequences within TW157 and TW570 are shown by black arrows. Genomic coordinates for the clusters are given in bases.

from 18 156 shotgun sequences (giving 8·9-fold coverage). Of these, 9960 were paired end reads from a pUC19 library with insert sizes of 3·0–3·3 kb, and 7276 were paired end reads from a pMAQ1b library with insert sizes of 5·0–5·5 kb. All identified repeats were bridged by read-pairs or end-sequenced PCR products. The sequence was assembled, finished, and annotated as described previously,[14] using Artemis to collate data and facilitate annotation. Pseudogenes had one or more mutations that would ablate expression; each of the inactivating mutations was subsequently checked against the original sequencing data. We manually checked potentially variable sequences against the original sequencing data. Variations were only considered for subsequent analysis if each of the different bases was clearly present in the sequences from at least two independent shotgun clones. We investigated metabolic pathways with the KEGG database.

Much of the usual chromosomal GC bias[15] was obscured by the anomalous nucleotide biases of the large non-coding regions. However, we were able to place the likely origin of replication near the first base of the *dnaA* gene, the usual point of chromosomal replication initiation in actinomycetes,[16] and this site was therefore chosen as the start of the sequence. SignalP and TMHMM were used to detect proteins likely to be secreted in, or localised to, the cell envelope. Protein clustering was done as previously described.[17] We initially identified and delineated *T whipplei* surface protein (WiSP) family β-strand repeats with Dotter for self-self comparisons, and HMMER (version 2.2g) was used to build a hidden Markov model. We then used this model to search for further copies of the repeat. Potentially phase-variable genes were identified by searching the genome for single base-pair tandem repeats of 9 bp or more (homopolymeric tracts), and tandem repeats of greater than 2 bp with five or more units (heteropolymeric tracts). We further assessed candidates for the likely effect of the tandem repeats on the translation of the associated gene. No significant heteropolymeric tracts were identified.

The sequence and annotation has been included in the GenBank, EMBL, and DDBJ databases with the accession number BX072543. The full annotation and additional references for some of the methods, along with further information and updated annotation are available from http://www.sanger.ac.uk/Projects/T_whippelii.

Role of the funding source
The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit the manuscript for publication.

Results

Figure 1 and panel 1 show the general features of the sequence of the *T whipplei* genome, which is 925 938 bp in size and has a G+C content of 46·3%. The genome contains 784 coding sequences, including only one identifiable pseudogene.

More than 5% (46 899 bp) of the chromosome is made up of non-coding repetitive DNA, which has a greatly biased dinucleotide content by comparison with the rest of the genome. This large amount of non-coding repetitive DNA accounts for a fairly low coding density (84·4%) for this organism. The non-coding DNA is located in two clusters, termed repeat cluster 1 (RC1) and repeat cluster 2 (RC2). RC1 is 45 299 bp long with a coding density of just 51·3%, and RC2 is 47 273 bp long with a coding density of 37·8%. RC1 and RC2 are located almost opposite one another on the chromosome map (figures 1 and 2). More than half the coding sequences within RC1 and RC2 encode membrane proteins.

We ascribed a putative function, based on sequence similarities with entries in public databases, to 74% of coding sequences. We designated 84 (11%) coding sequences as conserved hypotheticals, and predicted that most of the remaining 116 (15%) sequences, with no match in the databases, encode cell-envelope or secreted proteins. Of the *T whipplei* predicted proteins with no significant match in the public databases, most (86) are probably exported from the cell cytoplasm and are localised to the cell envelope.

Clustering analysis of all proteins encoded by the genome revealed a prominent family of predicted surface proteins termed WiSP. Alignment and analysis of WiSP sequences revealed a heterogeneous family of proteins with several identifying features (figure 3). Sizes of WiSP proteins range from 103 to 2308 residues. Some of the smaller members could be remnants of larger coding sequences and might not actually be expressed or be functional. It is noteworthy that RC1 and RC2 contain two and one WiSPs, respectively. Ten of the 14 family members have N-terminal secretion signal sequences. In eight cases the signal sequence forms part of an about 300-residue conserved domain (WiSP N-terminal domain; WND), which differs by only one or two aminoacids between different members. The domain is rich in serine and threonine residues and is likely to have a low complexity structure. Five of the 14 contain one or more predicted transmembrane domains near the C-terminus, which could potentially anchor the proteins in the bacterial membrane. TW774 is comprised of a single domain that is similar to the C-terminus of TW113 (94% identity over 120 aminoacids) and TW776 (96% identity over 47 aminoacids).

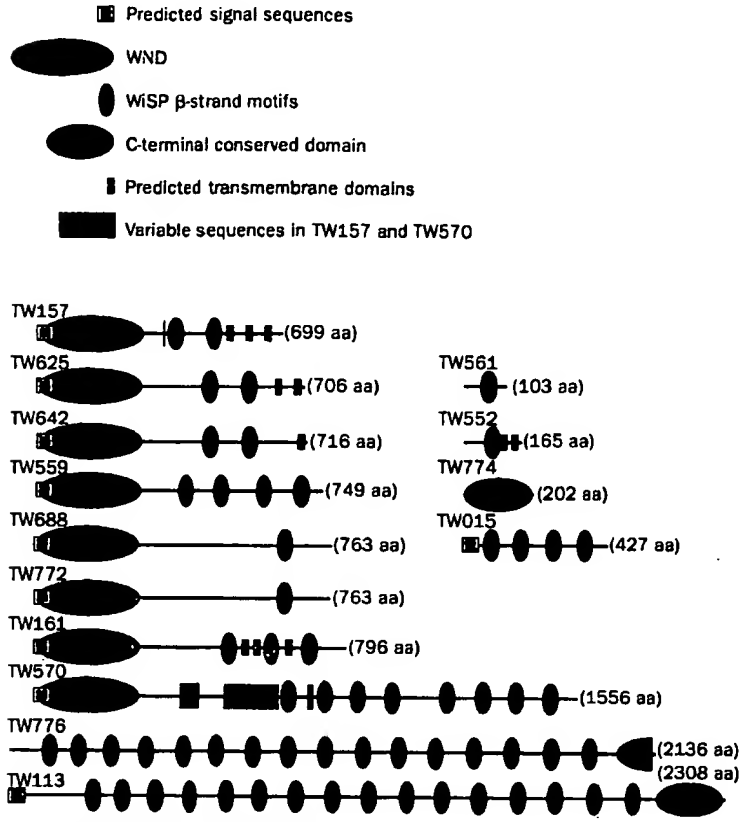

Figure 3: **Architecture of WiSP family**

With the exception of TW774, all WiSPs contain at least one copy of a β-strand motif. An alignment of all of these β-strand motifs is shown in webfigure 1 (http://image.thelancet.com/extras/03art1087webfigure1.pdf). TW113 and TW776 are comprised almost entirely of β-strand motifs, each having 16 copies. WiSP β-strand motif repeats have a median repeat length of 107 residues (mode 89 residues), but only the central portion (about 35 residues) is strongly conserved. The hidden Markov model used to identify these motifs was used to search the SWISS-PROT and TrEMBL protein databases for similar structural sequences. We identified similarities in long proteins of similar organisation—eg, the biofilm-associated protein, Bap, from *Staphylococcus aureus*. Homology searching also revealed similarities to HYR and PKD domains, both Ig-fold structures.

Five genes in *T whipplei* seem to be phase variable—ie, they can be randomly switched on and off by variation in short repeat tracts (panel 2); four are probably integral membrane proteins, and the fifth is TW642, a member of the WiSP group. That this mechanism is active is supported by the fact that three of these coding sequences are frameshifted at the repeat tract (in the off state), and that one of the repeats (downstream of the 5S rRNA) is variable in chromosomal PCRs of *T whipplei* DNA from different patients (data not shown).

Shotgun sequence assembly revealed 48 loci in the genome where alternative sequences existed in the shotgun clones (table); 37 of these were single nucleotide polymorphisms. All but one, a variable length tandem repeat, were located within WiSP coding sequences TW157 or TW570 (three and 44 variable loci, respectively). The effect of most of the variations was to change a single aminoacid. Only two of the variations, both in TW570, resulted in a frameshift mutation. For both TW157 and TW570, the sequence variation was localised. The three single-base variations in TW157 affect two neighbouring codons whereas the 44 loci in TW570 span 338 codons in three clusters (figures 2 and 3). In neither case do the variations affect the WiSP conserved domains. PCR amplification and sequencing of the N-terminal region of TW570 from other passages of the *T whipplei* culture used for sequencing, and from other cultures, also revealed a similar pattern of localised specific variation both within the PCR products and between the products and the final genomic sequence (see webfigure 2 http://image.thelancet.com/extras/03art1087webfigure2.pdf). Different variations predominated at different passages of the same culture. The two genes showing this variation are embedded within each of the repeat clusters described above (figure 2). Although most of the repetitive DNA in these clusters is non-coding, some of the short DNA repeats are identical to sequences located within the two genes, at the regions at which variation arises. For example, a 205 bp sequence, covering the most C-terminal three variants in TW570 is repeated within a non-coding region of RC2 29 kb upstream. The sequences within the non-coding repeat exactly duplicate the minority variants in the TW570 coding sequence (see webfigure 3 http://image.thelancet.com/extras/03art1087webfigure3.pdf).

Panel 2: Potentially phase-variable genes

Coding sequence	Repeat	Location within gene	State	Product
TW113	C (11)	N-terminal	Frameshifted	Integral membrane protein
TW159	C (11)	Promoter?	Intact	Integral membrane protein
TW447	C (11)	N-terminal	Frameshifted	Integral membrane protein
TW687	C (8)	N-terminal	Intact	Integral membrane protein
	C (10)		Non-coding	Downstream of 5S rRNA
TW642	C (11)	N-terminal	Frameshifted	WiSP protein

Location (coding sequence, codon)	Sequence variants	Aminoacid variants
TW157, 322	C or A	Ser or Tyr
TW157, 323	A or G	Ser or Gly
TW157, 323	T or C	**Ser**
TW570, 710	ACA or GGG	Thr or Gly
TW570, 705	C or A	Thr or Lys
TW570, 699–701	AACCAA or CTC	ThrThrAsn or ThrSer
TW570, 630	C or A	Arg or Ser
TW570, 620	T or C	Tyr or His
TW570, 617	T or G	Ile or Arg
TW570, 615	T or G	**Ser**
TW570, 615	C or A	Ser or Tyr
TW570, 615	T or G or A	Ser or Ala or Thr
TW570, 613	G or T or C	Gly or Val or Ala
TW570, 587	A or C	Thr or Pro
TW570, 584	A or T	Thr or Ser
TW570, 565	A or T	Thr or Ser
TW570, 557	T or A	Val or Glu
TW570, 548	A or C	Thr or Pro
TW570, 546	TT or TCCCT	Leu or SerLeu
TW570, 544	G or A	Gly or Glu
TW570, 544	G or A	Gly or Arg
TW570, 543	G or A	Arg or Lys
TW570, 542	C or A	Thr or Lys
TW570, 541	T or C	Asp or Asp
TW570, 541	A or G	Asp or Gly
TW570, 541	G or A	Asp or Asn
TW570, 538	C or T	Tyr or Tyr
TW570, 537	A or C	Ile or Ile
TW570, 504–506	CCGTGTTT or GCACAGAG	AlaValPhe or GlyThrGlu
TW570, 500–501	CAAGT or TATGG	ProSer or LeuSTOP
TW570, 497–498	GGTGGT or AAGACC	GlyGly or ArgThr
TW570, 493	C or G	Ala or Gly
TW570, 493	G or A or T	Ala or Thr or Ser
TW570, 492	C or T	Thr or Ile
TW570, 428	G or A	Arg or Gln
TW570, 423	T or A	Ser or Thr
TW570, 421	G or C	Lys or Asn
TW570, 420	C or A	Asp or Glu
TW570, 420	G or A	Asp or Asn
TW570, 398	C or AA	Frameshift
TW570, 393–394	CC or TA	ThrHis or ThrAsn
TW570, 386	CC or CAAA or CATATACCCAATATCTATC	Pro or frameshift or frameshift
TW570, 384	C or T	**Ser**
TW570, 379	G or A	Ala or Thr
TW570, 376	A or T	Thr or Ser
TW570, 374	GG or GGTG	Gly or frameshift
TW570, 372	A or T	Ile or Leu
TW600, 37-57	5, 6, or 7× CTAGAACTA	5, 6 or 7× LeuGluLeu

Synonymous changes in bold.

Aminoacid changes caused by variable sequences

Metabolic reconstruction shows that there are no genes for arginine, tryptophan, and histidine biosynthesis, and there are deficiencies in the ability to synthesise glycine, serine, leucine, and cysteine. Genes for synthesis of biotin, thiazole, and thiamine are absent, as are those for the fumarate reductase and the NADH dehydrogenase complexes. To enable import of essential compounds, *T whipplei* encodes 47 transport proteins, 33 of which are ABC family transporters. Although it is often difficult to predict a specific transport substrate, there is a possible operon of five coding sequences for an aminoacid import system (TW200–204). Oxidative phosphorylation seems to be limited to cytochrome oxidase and ATP synthase. Although glycolysis or gluconeogenesis, pentose phosphate pathway, and pyruvate metabolism seem intact, there is a complete absence of genes for the tricarboxylic acid cycle. The *T whipplei* genome encodes 20 (2·56%) proteins with predicted regulatory function (figure 4).

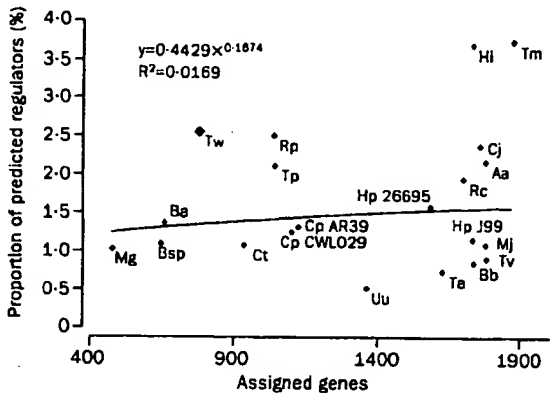

Figure 4: **Proportion of coding sequences that encode predicted regulators, as a function of genome size**

Data from genomes with less than 2000 coding sequences were obtained from the TIGR Comprehensive Microbial Resource (http://www.tigr.org/tigr-scripts/CMR2/CMRHomepage.spl), using the CMR automated annotation to ensure uniformity. Smaller genomes were selected so as to complement an earlier analysis[27] and provide a more relevant data set with which to compare the *T whipplei* genome. A best fit of the data is shown as a curve described by the equation $y=0{\cdot}4429X^{0{\cdot}1674}$, $R^2=0{\cdot}0169$. Mg=*Mycoplasma genitalium* G37; Bsp=*Buchnera* sp APS; Ba=*Buchnera aphidicola*; Tw=*Tropheryma whipplei* (in orange); Ct=*Chlamydia trachomatis*; Tp=*Treponema pallidum*; Rp=*Rickettsia prowazekii*; Cp=*Chlamydophila pneumoniae*; Uu=*Ureaplasma urealyticum*; Hp=*Helicobacter pylori*; Ta=*Thermoplasma acidophilum*; Rc=*Rickettsia conorii*; Hi=*Haemophilus influenzae*; Bb=*Borrelia burgdorferi*; Cj=*Campylobacter jejuni*; Aa=*Aquifex aeolicus*; Mj=*Methanococcus jannaschii*; Tv=*Thermoplasma volcanium*; Tm=*Thermotoga maritima*.

With respect to DNA repair, the genome only includes coding sequences for a Rec recombination pathway (*recA*, *recF*, *recG*, *recN*, *recO*, *recR*) and the ABC excision excinuclease (*uvrBAC*).

An analysis of coding sequences revealed 14 genes with anomalous dinucleotide content, codon use, or positional base preference (panel 3). Of these, 11 are WiSP proteins.

Panel 3: Genes with pronounced nucleotide anomalies

Coding sequence	Protein	Anomalous		
		Dinucleotide content	Codon usage	Positional base preference*
TW123	WiSP			
TW157	WiSP			
TW161	WiSP			
TW350	Coiled-coil			
TW391	unknown			
TW548	WiSP			
TW570	WiSP			
TW621	Pro/Ala-rich integral membrane			
TW665	WiSP			
TW842	WiSP			
TW868	WiSP			
TW772	WiSP			
TW774	WiSP			
TW788	WiSP			

*Clustering of position G+0 centred within codons.

Discussion

Our findings indicate that the genome of *T whipplei* has several novel features. First, the bacterium has an unexpectedly small genome, bearing the traits of strictly host-adapted organisms, including pronounced deficiencies in energy metabolism and requirements for external aminoacids. Second, despite the small genome size, the bacterium devotes a large amount of coding capacity to biosynthesis of surface-associated features, suggesting that interaction with its host plays a major part in the organism's lifestyle. Third, the genome reveals mechanisms for generating genetic variability, including phase variation and seemingly directed point mutations. Large regions of non-coding repetitive DNA, unlike anything previously seen in bacterial genome sequences, seem to be a central feature. Most of the variation generated seems to be directed towards changes in cell-surface proteins, indicating that these mechanisms have been developed to evade the host's immune response during the course of chronic disease.

Actinomycete genome sizes range from 1 million bp to 8 million bp[16] and generally have a high G+C content. At just under a million bp, the *T whipplei* genome is the smallest to be completely sequenced and, atypically, has an average G+C content of only 46%. It is noteworthy that the genome contains 784 coding sequences, including only one identifiable pseudogene. This low degree of gene disruption could in part be due to the total absence of mobile genetic elements, such as insertion sequences, within the genome. As bacterial genome size decreases, the proportion of coding sequences dedicated to housekeeping functions increases, as is the case with this bacterium.[16]

Genome analysis indicates that *T whipplei* has many cell-envelope genes typical of actinomycetes. A peptidoglycan biosynthesis gene cluster (TW540–TW551), for example, is largely similar to that seen in other sequenced actinomycete genomes. As with both *Mycobacterium tuberculosis* and *Streptomyces coelicolor*, a class B high-molecular-weight penicillin-binding protein/transpeptidase gene (TW548) is located towards the start of the cluster and the *ftsW* and *ftsQ* cell division genes towards the end. Unlike *M tuberculosis* and *S coelicolor*, the *ftsZ* gene, although present in the *T whipplei* genome, is not linked to this cluster.

The genome contains a gene cluster that seems to direct the biosynthesis and export of extracellular polysaccharide (TW032–042), which could explain a previous observation that the *T whipplei* cell wall includes, in addition to peptidoglycan, an unusual inner layer comprised mainly of polysaccharides.[3] This polysaccharide layer accounts for the positive periodic acid-Schiff (PAS) staining reaction, which is a major diagnostic feature in the histopathology of Whipple's disease. De-novo fatty acid biosynthesis seems to occur via the FasII route—ie, using a series of dissociated enzymes and a small acidic acyl carrier protein (ACP) rather than the multifunctional FasI polypeptide that is common to mycobacteria and corynebacteria. Genes encoding most of the FasII component proteins are clustered together (TW515–519), and there is no evidence for mycolic acid production.

15% of the *T whipplei* predicted proteins, and 74% of proteins with no match in the database, are thought to be exported from the cell cytoplasm and are localised to the cell envelope. The assignment of such a high proportion of *T whipplei*-specific proteins to the cell membrane or wall might reflect the importance to the organism of host interactions.

Our findings indicate that the WiSP proteins encoded in the *T whipplei* genome contain structural sequences similar to those identified in long proteins of similar organisation, some of which have been implicated in pathogenesis and immune evasion. One example is the biofilm-associated protein, Bap, from *Staphylococcus aureus*.[20] The Bap core region represents 52% of the protein and consists of 13 successive nearly identical repeats, each containing 86 aminoacids. The protein projects out from the cell surface, allowing the amino terminus to interact with neighbouring surfaces. We also noted similarities between WiSP β-strand motifs and HYR and PKD domains, both Ig-fold structures; HYR is thought to be involved in cellular adhesion.[21] Taken together, these data suggest that the WiSPs are surface proteins involved in host interaction, at least some of which are anchored in the membrane at the C-terminus, with the Ig-fold β-strand domains being structural elements, projecting the N-terminal domains out from the cell surface.

DNA sequence variation within the *T whipplei* genome is apparent at two levels, both of them associated with surface structures, and therefore potentially important in host interaction or immune evasion. First, *T whipplei* probably uses a process called slipped-strand mispairing to vary the expression of a small number of surface proteins (panel 2). This process, which has been well studied in several other pathogens[22] involves the random variation, during replication, in the length of short tandem repeat sequences, which consequently alters the transcription or translation of the genes containing them, randomly switching protein production on and off. This mechanism leads to phase variation, the random reassortment of this fraction of the complement of surface proteins. Four membrane proteins and one WiSP protein seem to be subject to this mechanism.

The second type of base-pair level variation indicated by our findings is an unusual hypervariation in the coding sequences of two WiSP proteins. Variation was not unexpected, since the culture used to make the shotgun libraries was grown over 17 months through 15 passages. What was surprising was the isolated distribution of the variations. Our results suggest that the large non-coding repeat regions of the genome are involved in generation of the variation in these genes, perhaps by acting as sources of sequence variants that could be copied into the expressed genes by some type of gene conversion event. In view of the difficulty of culturing *T whipplei*, the variations seen could be a consequence of a heterogeneous starting population. However, even if this were the case, the frequency of the variation seen and the fact that the variation seems to be constrained to specific regions and varies though culture passage, strongly imply that it is the result of a specific mechanism. Whatever this mechanism, the result is another level of variation generated in the WiSP proteins, underlining their likely importance in host interaction or immune evasion.

Many other factors indicate that *T whipplei* is a highly host-adapted organism. First, some global features of the genome are reminiscent of the genomes of obligate symbionts and obligate intracellular pathogens, such as members of the genera buchnera and rickettsia, respectively.[23,24] *T whipplei* has a small genome size for an actinomycete. Direct comparison with the complete genome sequences of related organisms such as those of *M tuberculosis* and *Corynebacterium diphtheriae* indicates that the small size is most likely due to gene loss (data not shown). Contraction of genome size due to gene decay

and loss is an important theme in the evolution of host-obligate genomes as the organism adapts to life in a less variable environment.[11,23] Another common feature of chromosomes from host-dependent bacteria is a reduction in G+C content, by comparison with the chromosomes of free-living close relatives.[11] Actinobacteria are characterised as high G+C bacteria, but *T whipplei* has a G+C content of just 46·3%. Reduction in G+C content might be associated with a loss of DNA repair functions,[25] as seems to be the case for *T whipplei*.

The absence of genes required for prototrophic growth is another indicator of reliance by a bacterium on a host for essential compounds.[24] Metabolic reconstruction of *T whipplei* indicates the absence of proteins for several key biochemical steps—eg, aminoacid biosynthesis. Aminoacids that cannot be synthesised must be scavenged from an extracellular source, namely the host. The *T whipplei* genome also suggests deficiencies in cofactor biosynthesis, energy metabolism, and carbohydrate metabolism. These findings suggest that *T whipplei* has become adapted to, or dependent on, its host.

Anomalies in G+C content, dinucleotide frequency, or codon usage could indicate genes or regions that have been acquired by recent horizontal transfer, and may reflect the frequency with which a bacterium has contact with other bacteria.[26] However, none of the 14 genes with nucleotide anomalies in the *T whipplei* genome were adjacent in the genome, and none lay in specific regions with other characteristics suggestive of horizontal transfer. Thus, we conclude that the observed compositional biases are not indicative of horizontal transfer, but reflect other properties, such as biased aminoacid use in the proteins. This lack of evidence for recent horizontal transfer, together with a complete absence of mobile elements in the genome, such as insertion sequences or prophage, is consistent with the notion that the organism resides in a secluded niche, and is not often exposed to foreign bacterial DNA.

One genome feature, however, lends support to a contrary argument and suggests that *T whipplei* senses (and is exposed to) a wider variety of environmental cues. In general, the proportion of genes that encode predicted regulatory factors rises with increasing bacterial genome size.[27] However, there is variability in the proportion of regulator genes among organisms with similar genome size. There is speculation that organisms exposed to greater numbers of environmental conditions require a larger number of regulators.[27] The *T whipplei* genome encodes a high proportion of proteins with predicted regulatory function, suggesting that the bacterium persists in a complex set of environmental niches. Although these could, of course, be alternative sites within the host, *Borrelia burgdorferi* also persists in many host sites and its genome contains a low proportion of predicted regulator genes.

Genes with clear predicted roles in *T whipplei* virulence are infrequent. Two predicted surface proteins TW583 and TW720 are similar to a protein, TadA, required for pilus-mediated tight adherence by the Gram-negative pathogen *Actinobacillus actinomycetemcomitans*.[28] TadA is widely distributed among bacteria and archaea. In *A actinomycetemcomitans*, it is a type IV secretion ATPase that probably energises the secretion and assembly of Flp pili.[29] As in *A actinomycetemcomitans*, the *T whipplei* TadA coding sequences are the first genes in operons of membrane protein encoding genes. The presence of two homologues in the stripped down genome of *T whipplei* suggests they have special importance for this organism.

Acquisition of iron is crucially important for bacterial pathogens in the iron-depleted host environment. For *T whipplei* ferri-siderophore uptake is encoded by a cluster of genes, which include two similar putative lipoprotein receptors. The remaining genes in the cluster encode an ABC transporter with similarity to the enterochelin permease of *Escherichia coli*. Although a homologue of the mycobacterial iron-dependent regulatory protein IdeR is present, no genes with significant homology for known siderophore biosynthesis genes are apparent, suggesting that *T whipplei* might only be able to scavenge xenosiderophores.

Determination of the *T whipplei* genome sequence is a major step forward in the characterisation of this previously poorly understood organism. The information gained from sequencing the *T whipplei* genome, both at the DNA and the protein level, can be used to design diagnostic tests for PCR or serological detection. There is evidence that Whipple's disease is underdiagnosed, so more sensitive tests for *T whipplei* will be a key to uncovering previously unrecognised manifestations, thus improving understanding of the disease.[30]

Contributors

J Parkhill was responsible for overall experimental design and analysis of sequence data. S D Bentley, B Barrell, and M Pallen contributed to sequence analysis and interpretation. C A Yeats did the domain analysis of the WiSP proteins. L Dover and G S Besra contributed to the analysis of the cell envelope components. M A Quail and H T Norberteczak generated libraries for sequencing and contributed to finishing strategies and PCR analysis. L D Murphy and D E Harris were responsible for the sequencing, finishing, and PCR analysis of the genome. A Goble, S Rutter, R Squares, and S Squares contributed to the sequencing and finishing experiments. A von Herbay provided the original clinical specimen from which the sequenced strain was isolated, and reviewed the manuscript; M Maiwald established the sequenced strain in continuous laboratory culture, prepared the DNA used for library construction, and contributed to manuscript preparation; D A Relman contributed to overall strategy for laboratory propagation of the sequenced strain and DNA preparation, assisted with annotation, and had responsibility for manuscript preparation.

Conflict of interest statement

None declared.

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Editorial: The Whipple Bacillus Lives (Ex Vivo)!

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Cultivation of the bacillus associated with Whipple's disease, *Tropheryma whippelii*, has been an elusive goal for many generations of clinicians and microbiologists familiar with this disease. The desire to identify this enigmatic organism has motivated many of these efforts. Many purported successes have later proven erroneous, and many more unsuccessful attempts have never been reported [1]. Cell-free media, animal cells, and animals themselves have all been used, resulting in recovery of a wide range of bacterial species, including members of the *Corynebacterium, Streptococcus, Propionibacterium*, and *Haemophilus* genera. The rough resemblance of *Rhodococcus equi*–, *Mycobacterium paratuberculosis*–, and *Mycobacterium avium* complex–associated diseases in foals, cows, and humans, respectively, to Whipple's disease has been noted; however, pathology closely mimicking that of the latter has never been knowingly and intentionally transferred to another human nor reproduced in a nonhuman host. Despite the unusual cell wall features of this bacillus and its reactivity to the periodic acid–Schiff (PAS) reagent [2], the absence of a specific microbial signature has greatly hindered efforts to evaluate these previous cultivation efforts.

Over the past 10 years, there has been a fundamental change in the approach to microbial identification and taxonomy. This change involves a decreasing reliance on cultivated organisms and their associated phenotypes, such as morphology, antigenicity, and biochemical activities, and an increasing reliance on genotype, that is, nucleic acid sequences [3, 4]. Certain genes, such as that of the small subunit ribosomal RNA (ssu rDNA), accurately reflect the evolutionary history of the entire genome and allow one to determine the relationships of any given organism with all others. By taking advantage of interspersed, highly conserved portions of these genes, one can amplify the intervening, phylogenetically useful sequence directly from infected clinical specimens and identify a previously uncharacterized or novel microbial pathogen [5, 6]. With this "broad-range polymerase chain reaction" (PCR) method, a unique, previously unrecognized bacterial ssu rDNA sequence was amplified from multiple independent Whipple's disease tissues [7, 8]. Phylogenetic analysis of this sequence suggested that the Whipple bacillus is an actinomycete and prompted the proposal of a new taxon, *Tropheryma whippelii* [8]. The *T. whippelii* ssu rDNA sequence now provides the basis for a specific PCR detection assay [8–11]. Armed with this diagnostic tool, Schoedon et al. [12] have tested a clever approach for in vitro Whipple bacillus propagation. The outcome of host infection depends in part upon a complex, local interplay of immune effector cells and cytokines. Pathogens often manipulate these host immune responses to render the local environment more hospitable and to enhance their survival or dissemination [13]; one strategy is to alter the local Th1/Th2 helper T cell profile. Suppression of tumor necrosis factor-α or interferon (IFN)-γ–mediated macrophage activation is a common strategy for microorganisms that choose an intracellular niche. Might one mimic this strategy by treating macrophages with cytokines or hormones that deactivate microbicidal pathways but preserve phagocytosis and thereby promote replication of an organism in a protected intracellular compartment? Interleukin (IL)-4, IL-10, and dexamethasone have been shown to enhance intracellular growth of certain pathogens within human macrophages by suppressing both oxidative and nonoxidative killing mechanisms but without inhibiting bacterial uptake [14, 15]. Schoedon et al. have taken this same approach for cultivating the Whipple bacillus. In this issue of the *Journal*, they provide evidence that *T. whippelii* replicates in the laboratory within human peripheral monocyte–derived macrophages, as well as within a macrophage-like cell line, when these host cells are treated with IL-4 [12].

The potential ramifications of these findings by Schoedon et al. are extensive. Yet, given the long and frustrating history of this disease and organism, one must evaluate this report carefully. In the absence of direct bacterial quantification and any obvious extracellular growth in vitro, how strong is the evidence for microbial replication? The authors relied on two types of data: (1) an increase in both the percentage of cells with visible PAS-positive inclusions and in the number of inclusions per cell and (2) PCR-based detection of *T. whippelii* DNA sequences after a number of cell passages sufficient to eliminate DNA detection after an equivalent inoculum dilution alone, that is, 100- to 1000-fold (in the absence of host cells). In theory, the first type of data might be explained in part by more rapid death of uninfected host cells (leading to an increase in the percentage of PAS-positive cells), although this seems unlikely, and by intracellular redistribution and trafficking of PAS-positive bacterial cell wall. PAS reactivity is difficult to quantitate and is only an indirect marker of bacterial number.

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However, quantitative PCR methods with internal standards would provide a more direct and reliable assessment of bacterial growth in this situation [16].

The exact identity of the organism propagated in this study is another crucial issue. Heart-valve tissue with apparently typical Whipple's disease pathology, from 2 persons, served as the inocula for cultivation [12]. From each tissue sample, a partial bacterial 16S rDNA fragment was amplified using broad-range PCR, from which ~400 bp of sequence was determined. Although these sequences were nearly identical to the corresponding segment of the previously published *T. whippelii* 16S rDNA sequence [8], this amount of primary sequence information is less than desirable. At various stages of tissue cocultivation, a *T. whippelii* PCR-based assay was positive, but the details of assay specificity are not provided. For the purpose of species, and certainly strain, identification, a complete 16S rDNA sequence is probably a minimum requirement; in most cases, additional sequence information from more rapidly evolving genetic loci is necessary. Nonetheless, it appears quite likely that the organism propagated by Schoedon et al. is either *T. whippelii* or a close relative.

What have we learned about the Whipple bacillus and its host from the results of this study? Might we have anticipated these findings from information previously available concerning this organism and its associated disease? First of all, *T. whippelii* appears to have a particular affinity for human macrophages and macrophage-like cells. Perhaps it is no coincidence that this organism elicits a prominent macrophage response during natural infection. Whether or not *T. whippelii* warrants the designation as an "intracellular pathogen," however, is unclear. The cell cocultivation conditions chosen by Schoedon et al. may have biased the outcome toward intracellular survival and growth and may not have provided the bacterium with the extracellular conditions that it encounters and prefers in a susceptible host. The pathology of Whipple's disease is notable for numerous intact extracellular bacilli, with some undergoing binary fission; at the same time, most bacilli within macrophages are at least partially degraded [1]. The same observation was made by Schoedon et al. in vitro, despite the macrophage-inactivating effects of IL-4 [12]. Second, the Whipple bacillus is at least microaerophilic. Third, the requirement for IL-4 treatment of macrophages draws attention to the possibility of a host cellular immune defect and the potential role of a polarized Th2 cytokine profile. Bjerknes and colleagues [17, 18] have suggested that monocytes and macrophages from Whipple's disease patients exhibit deficient microbial degradation capabilities. On the other hand, the effects of IL-4 are pleiotropic and nonspecific; the link between a Whipple's disease host defect and IL-4 may be only indirect. In theory, IFN-γ and its receptor are also possible key players in host susceptibility to this disease. Finally, did phylogeny predict physiology? When the relevant branch of the evolutionary tree is robust, microbial phylogeny sometimes predicts preferred growth conditions; however, because there are few known close relatives of *T. whippelii*, such insights would have been difficult to discern.

In an era of decreasing reliance on cultivated organisms and increasing reliance on rapid and specific molecular or sequence-based methods for microbial characterization, what is the value of propagating an organism such as the Whipple bacillus in the laboratory? With phylogenetically useful sequence alone, microbial identification and evolutionary analysis are possible; predictions can be made regarding metabolic, biochemical, and virulence-associated activities and then further evaluated with consensus PCR and sequencing; growth state might be estimated from quantitative rRNA measurements; and compelling arguments can be developed for a role in disease causation [19]. To the degree that additional genome sequence information may be further revealing, one might "walk" the chromosome of an uncultivated microorganism beginning with the ssu rDNA [20]; it may even be possible to determine a complete genome sequence from such organisms with shotgun cloning methods and powerful sequence assembly algorithms. However, the advantages of a laboratory propagated organism are still substantial.

A viable microorganism, provided with relevant growth conditions, readily reveals its metabolic and virulence capabilities. Disease models and correlates of pathogenicity can be established. Laboratory propagation creates substantial amounts of pure microbial cell mass, with which serologic assays can be developed, monoclonal antibodies elicited, and chromosomal DNA easily prepared. From recombinant chromosomal libraries, virulence-associated genes can be isolated, and the molecular mechanisms of disease causation can be explored. Immunodominant antigens can be cloned and expressed. Diagnosis can then be based on whole cell– or recombinant antigen–based serologic assays or on specific immunochemical and immunofluorescent tissue hybridization. Microbial drug susceptibility can be assessed in vitro. Recombinant antigens may be protective for susceptible hosts.

In theory, all of these advantages can now be realized for *T. whippelii*. In practice, several issues will first need to be addressed. The organism propagated by these authors should be characterized in greater detail. Optimization of growth conditions leading to consistent, high-titer culture yields will be important. One approach might involve cell lines bearing transgenes or genetic defects that render them hypersusceptible to *T. whippelii* growth. Intracellular bacterial degradation needs to be minimized. And, of course, the findings reported herein need to be reproduced by others. But if this work is substantiated, Schoedon and colleagues will have made a key contribution to a fascinating 90-year saga in clinical microbiology. No microorganism is uncultivatible; the real issue is whether we are intelligent enough to understand the sometimes complex and intimate growth requirements of our prokaryotic cousins.

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Detection of three different types of '*Tropheryma whippelii*' directly from clinical specimens by sequencing, single-strand conformation polymorphism (SSCP) analysis and type-specific PCR of their 16S–23S ribosomal intergenic spacer region

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The 16S–23S rDNA intergenic spacer region of organisms identical with or closely related to '*Tropheryma whippelii*', the uncultivated causative agent of Whipple's disease, was analysed directly from 38 clinical specimens of 28 patients using a specific nested PCR followed by direct sequencing. As compared to the reference sequence in public databases, two novel '*T. whippelii*' spacer types were recognized. In the absence of DNA–DNA hybridization data it is uncertain whether the three types found represent subtypes of a single species or three different but closely related species. Methods were developed to detect all three variants by single-strand conformation polymorphism analysis and by type-specific PCR assays, thus allowing the screening of large numbers of specimens. Further studies may provide a clue to the possible associations between the type of infecting strain and the various clinical presentations of Whipple's disease.

Keywords: Whipple's disease, '*Tropheryma whippelii*' typing, 16S–23S ribosomal intergenic spacer region, sequence analysis, single-strand conformation polymorphism

INTRODUCTION

Comparative sequence analysis of the 16S rRNA gene (16S rDNA) allowed the hitherto uncultivated presumptive agent of Whipple's disease to be identified as an actinomycete bacterium constituting the novel but not yet validated taxon '*Tropheryma whippelii*' (Wilson *et al.*, 1991; Relman *et al.*, 1992). Further, the sequence of the intergenic spacer region between the 16S and the 23S rDNA as well as the 5′ end of the 23S rDNA have also been determined (Maiwald *et al.*, 1996).

Several diagnostic '*T. whippelii*'-PCRs that target parts of the 16S rDNA have been established (Relman *et al.*, 1992; Rickman *et al.*, 1995; Altwegg *et al.*, 1996; Dauga *et al.*, 1997). However, such systems may not discriminate between closely related species as illustrated by identical or almost identical 16S rRNA gene sequences of *Mycobacterium kansasii* and *Mycobacterium gastri*, *Mycobacterium malmoense* and *Mycobacterium szulgai*, or *Aeromonas trota* and *Aeromonas caviae*, respectively (Martinez-Murcia *et al.*, 1992; Roth *et al.*, 1998). Since the intergenic spacer is more variable than the flanking structural genes it has been widely used for species identification and particularly for subtyping purposes in many other bacterial groups (Gürtler & Stanisich, 1996). We have designed a nested PCR for the detection of the '*T. whippelii*' 16S–23S rDNA spacer region directly in clinical specimens. Sequencing of the resulting PCR products from nine independent Swiss patients with Whipple's disease revealed no spacer polymorphism at all (Hinrikson *et*

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Abbreviation: SSCP, single-strand conformation polymorphism.

The GenBank accession numbers for the sequences of the '*T. whippelii*' 16S–23S rDNA spacer types 2 and 3 are AF100950 and AF100951, respectively (type 1 represents the original '*T. whippelii*' spacer sequence as deposited previously in GenBank under accession no. X99636).

Table 1. Source and molecular characterization of the clinical specimens used for genotyping of '*T. whippelii*'

Symbols and abbreviations: + and −, positive and negative result; 1 to 3, '*T. whippelii*' spacer types 1 to 3, respectively; NT, not tested.

Patient no.	Specimen		16S rDNA PCR*	16S–23S rDNA spacer							
	Origin	Code		PCR†	Sequencing‡	SSCP	Type-specific PCRs§				
							A	B	C	D	E
1	Duodenum	5713/96	+	+	1	1	+	−	+	−	−
2	Heart valve	724/98	+‖	+	1	NT	+	−	+	−	−
	Heart valve¶	838/98	+	+	1	NT	+	−	+	−	−
3	Gastric aspirate	1124/97	+#	+	1	NT	+	−	+	−	−
	Duodenum	1125/97	+#	+	1	NT	+	−	+	−	−
	Duodenum	1125II/97	+	+	NT	1	+	−	+	−	−
4	Gastric aspirate	616/97	+	+	1	NT	+	−	+	−	−
	Gastric aspirate	616II/97	+	+	1	1	+	−	+	−	−
5	Small intestine	368/97	+#	+	1	NT	+	−	+	−	−
6	Cerebrospinal fluid	1613/97	+	+	1	1	+	−	+	−	−
	Cerebrospinal fluid	1615/97	+	+	1	1	+	−	+	−	−
7	Duodenum¶‖	459-98	+**	+	1	1	+	−	+	−	−
8	Duodenum¶‖	836-96	+**	+	1	1	+	−	+	−	−
9	Duodenum¶	4549-94	+**	+	1	1	+	−	+	−	−
10	Duodenum	725/97	+#	+	1	1	+	−	+	−	−
11	Ileum	168/97	+#	+	1	1	+	−	+	−	−
12	Duodenum	1908/96	+	+	1	NT	+	−	+	−	−
13	Synovial fluid	5794/96	+‖	+	1	NT	+	−	+	−	−
	Synovial fluid	5831/96	+‖	+	NT	1	+	−	+	−	−
14	Cerebrospinal fluid	141/97	+#	+	1	NT	+	−	+	−	−
15	Heart valve	214/96	+‖	+	1	1	+	−	+	−	−
16	Gastric aspirate	895/97	+	+	2	2	−	+	−	+	−
17	Synovial fluid	749/98	+‖	+	NT	2	−	+	−	+	−
	Intraoperative swab	875/98	+	+	NT	2	−	+	−	+	−
18	Ileum	2249/96	+	+	2	2	−	+	−	+	−
19	Small intestine	5996/96	+#	+	2	2	−	+	−	+	−
20	Duodenum	604/97	+#	+	NT	2	−	+	−	+	−
21	Gastric aspirate	1037/97	+#	+	2	2	−	+	−	+	−
22	Intervertebral disc	3994/95	+‖	+	NT	2	−	+	−	+	−
23	Heart valve	271/98	+‖	+	2	2	−	+	−	+	−
24	Duodenum	771/97	+#	+	2	2	−	+	−	+	−
	Ileum	906/97	+#	+	2	2	−	+	−	+	−
25	Heart valve	5194/95	+‖	+	2	2	−	+	−	+	−
26	Duodenum	530/97	+#	+	3	NT	−	+	−	−	+
	Gastric aspirate	532/97	+#	+	3	3	−	+	−	−	+
	Gastric aspirate	952/97	+#	+	3	NT	−	+	−	−	+
27	Duodenum	220/97	+#	+	NT	3	−	+	−	−	+
28	Gastric aspirate	672/97	+#	+	NT	3	−	+	−	−	+

*'*T. whippelii*'-specific amplification with primers TW-1 & TW-2 (Altwegg *et al.*, 1996).

†'*T. whippelii*'-specific nested PCR using primer pairs tws1 & tws2 followed by tws3 & tws4.

‡Analysis of products derived from '*T. whippelii*'-specific nested PCR using primer pairs tws1 & tws2 followed by tws3 & tws4.

§Primer combinations: A, twsA1 & twsB1; B, twsA2 & twsB2; C, twsA1 & twsC1; D, twsA2 & twsC1; E, twsA2 & twsC2.

‖Confirmed by sequencing of products derived from broad-range (universal eubacterial) amplification (Goldenberger *et al.*, 1997).

¶Paraffin-embedded tissue.

#Confirmed by sequencing of products derived from '*T. whippelii*'-specific semi-nested PCR with primer pairs TW-1 & TW-2 followed by TW-4 & TW-2 (Brändle *et al.*, 1999).

**Confirmed by sequencing of products derived from '*T. whippelii*'-specific amplification with primers analogous to TW-1 & TW-2.

al., 1999). Nonetheless, specimens from additional patients were investigated using the same approach.

In the present study, we describe two new 16S–23S rDNA spacer types of '*T. whippelii*' or closely related organisms. In addition, two new approaches, single-strand conformation polymorphism (SSCP) analysis and type-specific PCR assays, were developed that are much easier to perform than sequencing and thus allow the screening of large numbers of specimens.

METHODS

Specimens. A total of 38 clinical specimens from 28 patients shown to contain '*T. whippelii*' 16S rDNA by semi-nested PCR using primer pairs TW-1 & TW-2 followed by TW-4 & TW-2 (Brändle *et al.*, 1999) were investigated (Table 1). They included cerebrospinal fluids ($n = 3$), heart valves ($n = 5$), a biopsy of an intervertebral disc, synovial fluids ($n = 3$), intestinal biopsies ($n = 17$), gastric aspirates ($n = 8$), and an intraoperative swab from a joint. In 19 specimens representing 13 patients the identity of 16S rDNA amplicons (229 bp) had previously been confirmed by direct sequencing. For an additional eight specimens from eight patients comparable results had been obtained by broad-range (universal eubacterial) amplification and sequencing of a ±530 bp fragment (Goldenberger *et al.*, 1997). No sequence data were available for the remaining 11 samples. Negative controls consisted of intestinal biopsies ($n = 23$), a bowel aspirate, and gastric aspirates ($n = 17$) that were negative for '*T. whippelii*' by semi-nested PCR with the above primer pairs.

Extraction of DNA. Cerebrospinal fluids, synovial fluids, gastric aspirates, the 0·85% NaCl suspension (2 ml) of the intraoperative swab, and the bowel aspirate were centrifuged for 10 min at 14000 *g*. These pellets as well as the biopsies were suspended in digestion buffer (50 mM Tris/HCl, pH 8·5, 1 mM EDTA, 0·5% SDS, 200 µg proteinase K ml^{-1}) and incubated at 55 °C for 1 h 30 min with agitation. DNA was extracted with QIAamp DNA-binding columns (QIAGEN) according to the manufacturer's protocol except for the final step using only 100 µl (instead of 200 µl) of elution buffer AE. Five microlitres of the eluate were used for PCR.

PCR assays. Primers used for amplification and/or nested reamplification are listed in Table 2. Amplifications were done in a final volume of 50 µl containing 200 µM each deoxynucleotide, 2·5 U AmpliTaq Gold polymerase with the appropriate amount of its optimized buffer (Perkin-Elmer), 25 pmol each primer, 2% (v/v) Tween 20, and 5 µl DNA extract. Reamplification was identical, except that 1–5 µl of amplicon was used as template and Tween 20 was omitted. Each PCR run included molecular-grade H_2O (LAL reagent water; BioWhittaker Europe, Belgium) as negative control. All PCRs were performed on a GeneAmp PCR System 9600 (Perkin-Elmer) and started at 95 °C for 12 min. Amplifications and reamplifications with primer pairs tws1 & tws2 and tws3 & tws4 were done for 40 cycles at 95 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 1 min. Reamplifications with primer combinations (i) twsA1 & twsB1 (system A, targeting spacer type 1), (ii) twsA2 & twsB2 (system B, types 2 and 3), (iii) twsA1 & twsC1 (system C, type 1), (iv) twsA2 & twsC1 (system D, type 2), and (v) twsA2 & twsC2 (system E, type 3) were performed using 20 cycles at 95 °C for 1 min, at 70 °C for 1 min, and at 72 °C for 1 min. All PCRs ended at 72 °C for 10 min. PCR products (10 µl) were separated by electrophoresis on a 2% (w/v) agarose gel, stained with ethidium bromide, and detected under UV light.

Sequence analysis. Direct sequencing of products derived from nested PCR with primer pairs tws1 & tws2 followed by tws3 & tws4 was performed on an ALFexpress DNA Sequencer (Pharmacia Biotech). Amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. The cycle sequencing reaction was performed in both directions with the 5'-fluorescence-labelled primers tws3 or tws4, respectively, and the Thermo Sequenase Kit with 7-deaza-dGTP (Amersham). The cycling conditions were the same as described for the PCR assays except for an initial denaturation at 95 °C for only 5 min. Data analysis included

Table 2. Oligonucleotides used to analyse the 16S–23S rDNA intergenic spacer region polymorphisms of '*T. whippelii*'

Primer* /sense†	Sequence (5′ → 3′)	Position‡	'*T. whippelii*' target	
			rDNA	Spacer type(s)
tws1/f	ATCGCAAGGTGGAGCGAATCT	1213 → 1233	3′ end of 16S	1, 2 and 3
tws2/r	CGCATTCTGGCGCCCCAC	1940 → 1923	5′ end of 23S	1, 2 and 3
tws3/f	CCGGTGACTTAACCTTTTTGGAGA	1387 → 1410	3′ end of 16S	1, 2 and 3
tws4/r	TCCCGAGGCTTATCGCAGATTG	1875 → 1854	5′ end of 23S	1, 2 and 3
twsA1/f	AAGTGATACCGCCATAGTGCACTGT	1527 → 1551	16S–23S spacer	1
twsA2/f	AAGTGATACCGCCATAGTGCACTGC	1527 → 1551	16S–23S spacer	2 and 3
twsB1/r	CTCCCGTGAGCTTGTGCCCAAAAC	1600 → 1577	16S–23S spacer	1
twsB2/r	CTCCCGTGAGCTTGTGCCCAAAC	1600 → 1577	16S–23S spacer	2 and 3
twsC1/r	AATAGTGCACACAAGTGCATAAGCA	1667 → 1643	16S–23S spacer	1 and 2
twsC2/r	AATAGTGCACACAAGCGCATAAGCG	1667 → 1643	16S–23S spacer	3

* This study except tws1, tws2, tws3 and tws4 (Hinrikson *et al.*, 1999).

† f, forward; r, reverse.

‡ According to '*T. whippelii*' sequence, GenBank accession no. X99636 (Maiwald *et al.*, 1996).

the softwares ALFwin version 1.10 (Pharmacia Biotech) and the Genetic Computer Group (GCG) package (University of Wisconsin, Madison, USA) for comparison with entries in the GenBank/EMBL databases.

***HaeIII*-restriction and SSCP analysis.** The same QIAGEN-purified amplicons (1–3 µl) as used for sequencing were digested with 1 unit of endonuclease *Hae*III (restriction sites are indicated in Fig. 1) according to the provider's protocol (Boehringer Mannheim). Performance of digestion was checked by 2% agarose gel electrophoresis. Digests (1 µl) were mixed with denaturation buffer (4 µl) according to Widjojoatmodjo *et al.* (1994), incubated at 95 °C for 10 min, and stored on ice before loading onto a non-denaturating 10% polyacrylamide gel (CleanGel 36S; Pharmacia Biotech). Samples (2 µl) were separated with DNA Disc buffer (Pharmacia Biotech) on a 2117 Multiphor II Electrophoresis Unit (Pharmacia Biotech) at 4 °C using 100 V for 30 min followed by 400 V for 180 min, and DNA was visualized by silver-staining. Profiles were interpreted visually.

RESULTS

Amplification and direct sequencing of the 16S–23S rDNA spacer region

All DNA extracts were investigated by nested PCR with primer pairs tws1 & tws2 followed by tws3 & tws4 targeting the '*T. whippelii*' 16S–23S rDNA spacer and flanking coding regions. Amplicons of the expected size (~ 490 bp) were detected in all specimens previously shown to harbour '*T. whippelii*' 16S rDNA but none of the negative controls (results not shown). All amplicons that were sequenced ($n = 30$) perfectly matched the revised '*T. whippelii*' reference sequence (GenBank accession no. AF074933; Hinrikson *et al.*, 1999) in the 3′ and 5′ terminal regions of the 16S and 23S rRNA genes, respectively. However, sequence variability was repeatedly observed within the 16S–23S rDNA spacer sequence with the following five dimorphic sites: (i) T or C [position 1551 of original spacer reference sequence (Maiwald *et al.*, 1996)]; (ii) TTTT or ()TTT (positions 1578–1581); (iii) T or C (position 1619); (iv) T or C (position 1643); and (v) A or G (position 1652). According to these single-base alterations three spacer types were recognized (Fig. 1).

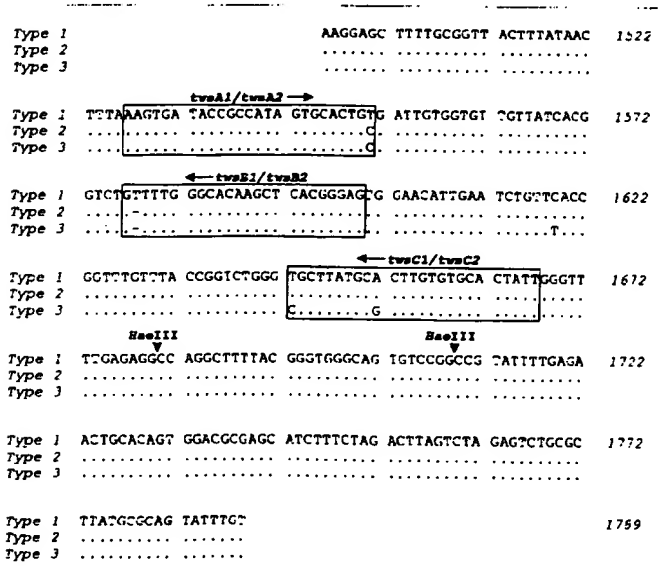

Fig. 1. 16S–23S rDNA intergenic spacer region variants of '*T. whippelii*'. The base numbering is given according to Maiwald *et al.* (1996); dots and hyphens symbolize identity and alignment gaps, respectively. Target regions of primers used for type-specific reamplification as well as restriction sites of endonuclease *Hae*III are indicated by bold type.

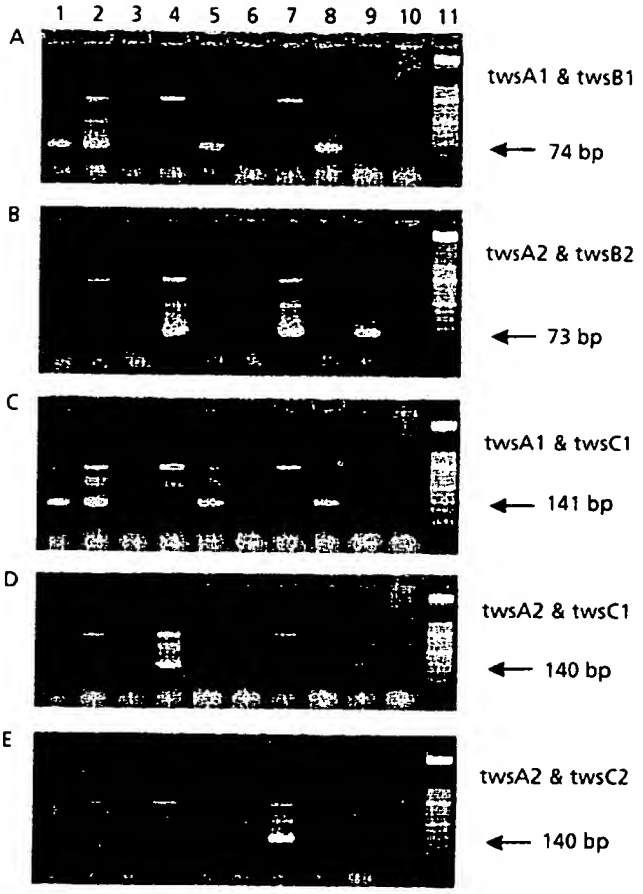

Fig. 2. Representative results of nested PCR assays for direct detection of '*T. whippelii*' 16S–23S rDNA spacer types in clinical specimens on ethidium bromide-stained agarose gels. PCR products derived from amplification using primer pair tws3 & tws4 were reamplified with various type-specific primer combinations: twsA1 & twsB1 (A, targeting spacer type 1), twsA2 & twsB2 (B, types 2 and 3), twsA1 & twsC1 (C, type 1), twsA2 & twsC1 (D, type 2), and twsA2 & twsC2 (E, type 3). The expected products are indicated with arrows. Lanes 1–10: Clinical specimens positive for '*T. whippelii*' spacer type 1 (lanes 1, 2, 5 and 8), spacer type 2 (lanes 4 and 9), and spacer type 3 (lane 7), and negative controls (lanes 3, 6 and 10). Lane 11, molecular mass marker (50 bp ladder; Boehringer Mannheim).

SSCP analysis

All 27 amplicons digested with *Hae*III yielded two fragments of the expected sizes (295/294 and 166 bp, respectively) by agarose gel electrophoresis while the remaining 29 bp fragments were not clearly visible

(results not shown). SSCP analysis using electrophoretic conditions optimized for the single strands of the 295/294 bp fragments (spanning the dimorphic sites) resulted overall in three distinct patterns (not shown). They all consisted of two upper (very closely migrating) bands (near the double-stranded 622 bp marker) and one lower band (between the double-stranded 622 and 527 bp marker). As compared to the profiles corresponding to spacer type 1, a reduced mobility of the lower band was characteristic for the patterns derived from fragments of spacer type 2; a decreased migration of both the lower as well as the upper bands has been detected for all the samples representing spacer type 3.

Type-specific PCR assays

All DNA extracts were amplified with primer pair tws3 & tws4 and directly reamplified in five separate reactions with primer combinations targeting dimorphic spacer sites (Fig. 2). All specimens known to contain '*T. whippelii*' 16S rDNA were classified according to their type-specific amplicons (74/73 bp and 141/140 bp, respectively), with type-specific reamplification systems A and C being positive for type 1, systems B and D for type 2, and systems B and E for type 3. None of these samples was positive for more than one spacer type (Table 1). The appearance of tws3 & tws4 amplicons and other minor products (>200 bp; representing amplicons derived from combinations of 1st and 2nd round primers) correlated with the amount of target DNA present in the clinical samples. All 41 controls tested remained negative for spacer products and did not show non-specific bands.

Frequency of the three spacer types in clinical specimens

Spacer type 1 was found in 15 patients, types 2 and 3 in 10 and 3, respectively (Table 1). Spacer types did not correlate with the type of specimen analysed.

DISCUSSION

The various clinical manifestations of Whipple's disease ranging from chronic low grade fever to life-threatening endocarditis (Dobbins, 1987) have often been assumed to represent differences between the infecting strains of '*T. whippelii*' or to be due to closely related, '*T. whippelii*'-like species. This, however, remained pure speculation as nobody has ever looked at such differences, mainly because of the absence of reliable cultures (Schoedon *et al.*, 1997). In this study, we have analysed the 16S–23S rDNA spacer sequences directly from 38 clinical specimens of 28 patients. These specimens had previously been shown to contain 16S rDNA of '*T. whippelii*' by a specific assay (Table 1). The identity of the respective amplicons from 19 samples of 13 patients had been confirmed by sequence analysis (Brändle *et al.*, 1999; Ehrbar *et al.*, 1999). For an additional eight specimens from eight patients comparable results had been obtained by broad-range (universal eubacterial) amplification and sequencing of a ± 530 bp fragment (Goldenberger *et al.*, 1997), thus excluding also the presence of significant amounts of DNA from unrelated bacteria.

We considered the analysis of the 16S–23S rDNA spacer region a promising approach to possibly detect strain differences as it has been shown for a variety of organisms that the spacer region is more variable than the flanking regions encoding 16S and 23S rRNA, respectively (Gürtler & Stanisich, 1996). In a first series of nine patients no variability had been found (Hinrikson *et al.*, 1999) and we, therefore, decided to analyse as many different samples as possible to verify these findings using the same approach, including sequence analysis of PCR products. This resulted in the detection of five dimorphic sites constituting three different spacer types (Fig. 1). In each of the eight patients with more than one positive specimen the same type was found in all independently processed samples (Table 1). The most frequent type detected, i.e. spacer type 1, perfectly matched the original '*T. whippelii*' spacer sequence (294 bp) previously deposited in GenBank (Maiwald *et al.*, 1996). As compared to this reference sequence, spacer types 2 and 3 differed solely at two and five nucleotide positions, respectively (Fig. 1). Nonetheless, these slight DNA alterations could be confirmed by SSCP analysis of *Hae*III-generated spacer fragments spanning all variable nucleotide positions found (not shown). As anticipated from the sequencing data, a total of three distinct SSCP profiles were obtained, each corresponding to one of the spacer types. Because both sequencing and, to some extent, SSCP analysis are tedious and time-consuming, we decided to establish type-specific PCR assays based on the polymorphisms found (Fig. 2). For each single specimen, the results of the five different assays were as expected (Table 1), thus confirming the reliability of each of the three methods used. While type-specific PCR is straightforward for detecting the presently known three types, SSCP and sequence analysis have the potential of recognizing additional types not related to the currently known five dimorphic sites.

In all specimens analysed only one single spacer type was detectable. Assuming that each patient is infected with only one single strain as suggested by the eight patients with multiple specimens, this may be explained by the presence of one single rRNA operon, i.e. spacer region, per strain as described for other actinobacteria (Gürtler & Stanisich, 1996). If so, any fixed single-base mutation in the rDNA spacer leading to an inappropriately folded molecule would have to be compensated by further sequence variation. Interestingly, the spacer types now found seem to have evolved mainly by pairwise DNA alterations (position 1551 ⇔ position 1578, and position 1643 ⇔ position 1652, Fig. 1) as indicated by the fact that five dimorphic sites (with 32 possible combinations) but only three different spacer types have been found.

The variations found raise the question whether they represent three different, closely related species or three subtypes of the single species '*T. whippelii*'. Partial 16S sequences determined for most specimens included in this study did not reveal any differences as compared to the published sequence. However, as has been shown for other organisms, even complete identity of 16S rDNA sequences does not prove species identity (Fox *et al.*, 1992) but suggests a very close relationship (Stackebrandt & Goebel, 1994). To solve the problem DNA–DNA hybridization studies are definitely needed but not feasible due to the fact that '*T. whippelii*' has not yet been cultured on artificial media. As long as hybridization data are not available, we suggest that the three types found be regarded as subtypes of the single species '*T. whippelii*' for practical reasons and because of the small number of variable nucleotides which is similar or even less than the variations found within other species (Gürtler & Stanisich, 1996).

We conclude that at least three different '*T. whippelii*' 16S–23S rDNA spacer types can be detected with different frequencies directly from human clinical specimens. Further studies including samples from other geographic areas as well as from environmental sources (Maiwald *et al.*, 1998) may show whether additional spacer types can be found. Such investigations are much easier to perform when using either type-specific PCR assays and/or SSCP analysis as compared to sequencing. Although an obvious association of spacer types and types of specimens analysed was not found, it remains to be shown whether the '*T. whippelii*' spacer types may be correlated with different clinical presentations of Whipple's disease or even with the possible carrier state for 'Whipple bacilli' as postulated previously (Ehrbar *et al.*, 1999).

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CULTIVATION OF THE BACILLUS OF WHIPPLE'S DISEASE

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ABSTRACT

Background Whipple's disease is a systemic bacterial infection, but to date no isolate of the bacterium has been established in subculture, and no strain of this bacterium has been available for study.

Methods Using specimens from the mitral valve of a patient with endocarditis due to Whipple's disease, we isolated and propagated a bacterium by inoculation in a human fibroblast cell line (HEL) with the use of a shell-vial assay. We tested serum samples from our patient, other patients with Whipple's disease, and control subjects for the presence of antibodies to this bacterium.

Results The bacterium of Whipple's disease was grown successfully in HEL cells, and we established subcultures of the isolate. Indirect immunofluorescence assays showed that the patient's serum reacted specifically against the bacterium. Seven of 9 serum samples from patients with Whipple's disease had IgM antibody titers of 1:50 or more, as compared with 3 of 40 samples from the control subjects ($P<0.001$). Polyclonal antibodies against the bacterium were generated by inoculation of the microorganism into mice and were used to detect bacteria in the excised cardiac tissue from our patient on immunohistochemical analysis. The 16S ribosomal RNA gene of the cultured bacterium was identical to the sequence for *Tropheryma whippelii* identified previously in tissue samples from patients with Whipple's disease. The strain we have grown is available in the French National Collection.

Conclusions We cultivated the bacterium of Whipple's disease, detected specific antibodies in tissue from the source patient, and generated specific antibodies in mice to be used in the immunodetection of the microorganism in tissues. The development of a serologic test for Whipple's disease may now be possible. (N Engl J Med 2000;342:620-5.)

WHIPPLE'S disease is a systemic bacterial infection characterized by fever, weight loss, diarrhea, lymphadenopathy, and polyarthritis and, occasionally, by cardiac manifestations such as myocarditis, pericarditis, and endocarditis[1,2] or by central nervous system involvement.[3] George Whipple described the disease in 1907,[4] and its bacterial origins were confirmed by electron microscopy in 1961.[5] The diagnosis is usually established on microscopy by the identification in a duodenal-biopsy specimen of infiltration by large macrophages with bacteria positive for the periodic acid–Schiff (PAS) stain.[6] In 1991 Wilson et al.[7] used broad-range primers to amplify and sequence a portion of the 16S ribosomal RNA gene of the bacterium for Whipple's disease, allowing classification of the bacterium within the Actinomycetes clade. These findings have been confirmed and extended.[8] Since then the polymerase chain reaction (PCR) has been reported to be a useful tool for the diagnosis of Whipple's disease.[9,10]

Culture of the bacillus has been an elusive goal for many generations of microbiologists.[11] In 1997 the bacterium was isolated and grown in human macrophages inactivated with interleukin-4.[12] However, that isolate could not be subcultured, and no isolate is currently available.[13]

We report the successful isolation and establishment of a strain of the bacterium for Whipple's disease obtained from the mitral valve of a patient with blood-culture–negative endocarditis, the generation of specific antibodies against the bacterium in mice, the detection of the bacterium in the patient's mitral valve by immunochemistry with these antibodies, and the detection of specific antibodies against the bacterium in the patient's serum.

METHODS

The Index Patient

A 42-year-old man with mental retardation owing to encephalitis as a child was noted to have clubbing and a heart murmur in the autumn of 1997. Apparently, he had also had rheumatic fever as a child, although this could not be confirmed. An echocardiogram showed a thickened aortic valve with severe insufficiency and normal left ventricular function. During the winter, worsening congestive heart failure developed, and the patient was hospitalized twice with weight loss and pneumonia. By May 1998, he had lost 15 kg in weight and was admitted to the hospital with nausea and vomiting. There was no history of diarrhea, no evidence of organomegaly on abdominal examination, and no lymphadenopathy. He was transferred to a hospital in Halifax, Nova Scotia, Canada, in May 1998. An echocardiogram demonstrated a vegetation on the anterior leaflet of the mitral valve and a small

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vegetation on the chordae of the anterior leaflet. There was associated mitral insufficiency with a flail anterior leaflet, severe aortic regurgitation, vegetations visible on the leaflets, and a small abscess of the annulus next to the septum. During surgery no aortic-valve tissue could be identified on gross examination, but there were masses of vegetations in the location of the aortic valve that were completely excised and replaced with a homograft. The patient recovered uneventfully and was sent home 14 days later while taking antibiotics. At a follow-up visit nine months later the patient remained well.

The surgically resected tissues were fixed in formalin or frozen at −80°C. Slices of paraffin-embedded tissue samples were cut 5 μm thick and stained with hematoxylin and eosin. The PAS stain and other stains were used to detect bacteria.[14]

Primary Isolation by Cell Culture

Culture was performed by the centrifugation–shell-vial technique with a human fibroblast cell line (HEL) that is used in our laboratory to detect intracellular bacteria, as previously described.[15,16] All cell lines and culture reagents are checked weekly for bacterial contamination. Frozen cardiac-valve tissue was placed in minimal essential medium and crushed, and the suspension was used to inoculate three shell vials (Table 1). The inoculated vials were processed as previously described.[15,16] The cultures were analyzed for bacteria by cytocentrifugation of 100 μl of the shell-vial supernatant followed by Gimenez staining[17] on days 10, 20, and 30. On day 30, the shell-vial supernatant and inoculated cells were harvested, inoculated into 25-cm² cell-culture flasks (flask 1) with 5 ml of medium, and incubated at 37°C in an atmosphere of 5 percent carbon dioxide. Every week for six weeks (until day 72), the cells were examined with an inverted microscope for cytopathic effects, and the incubation medium was replaced. Before the medium was replaced, 200 μl of the supernatant was obtained for cytocentrifugation and staining with Gimenez, Gram's acridine orange, Ziehl–Neelsen, and PAS stains.

Propagation of the Isolate

The isolate was propagated in HEL cells grown under previously described conditions (Table 1).[15,16] On day 75, 3 ml of supernatant from flask 1 was used to inoculate 10 shell vials, and 2 ml was used to inoculate a confluent monolayer of cells in a 25-cm² cell-culture flask (flask A) with 5 ml of medium. One of the shell vials was used to study generation time. The cells were harvested with the remaining supernatant and resuspended in fresh medium in order to obtain 10 ml of cell suspension, which was divided into five 2-ml aliquots. The cells of one aliquot were lysed by four cycles of freezing and thawing in liquid nitrogen and hot water (55°C) and inoculated onto confluent monolayers of cells in a 25-cm² cell-culture flask (flask C) with 5 ml of medium. Two aliquots were inoculated onto a confluent monolayer of cells in two 25-cm² cell-culture flasks (flasks B and D) with 5 ml of medium. On day 85, the medium in all flasks and shell vials was replaced by fresh medium. The cells in flask D were harvested and inoculated into a cell-free 75-cm² cell-culture flask (flask D2) with 15 ml of medium. Before the medium was replaced, 200 μl of each supernatant was obtained for cytocentrifugation and PAS staining. On days 95 and 105, the medium in all flasks and shell vials was again replaced. Small portions of the cell monolayers were scraped to obtain cell smears for PAS staining. The efficacy of propagation was evaluated by semiquantitative counts of these cell smears. Each smear was analyzed microscopically at a magnification of 1000 for PAS-positive bacilli. A score of 0 was assigned if no PAS-positive bacilli were found; a score of + indicated that bacilli were present but hard to find, a score of ++ indicated that bacilli were easily detected but were not present in all fields, and a score of +++ indicated that bacilli were present in all fields. All smears were evaluated in a blinded fashion by two investigators. To ensure continued production of the isolate, as soon as a flask was given a score of +++, the cells were harvested and inoculated into three 150-cm² cell-free cell-culture flasks, with the volume adjusted to 35 ml by the addition of fresh medium. We also attempted to propagate the isolate by inoculating the cells onto monolayers of MRC 5 cells cultured in the same way as were HEL cells and in axenic medium (chocolate agar and Columbia sheep's-blood agars, BioMérieux, Marcy l'Etoile, France) and incubated at 32° and 37°C in the presence of 5 percent carbon dioxide and in microaerophilic and anaerobic conditions. We also incubated the isolates with cell-culture medium alone and with cell-culture medium containing a lysate of HEL cells at 32° and 37°C in the presence of 5 percent carbon dioxide.

TABLE 1. SUMMARY OF THE ISOLATION PROCEDURE.

STUDY DAY	PROCEDURE
1	Inoculation of 3 shell vials
30	Passage of 1 shell vial in a 25-cm² cell-culture flask
65	Evidence of cytopathic effect and microorganisms
72	Detection of growth (PCR-positive)
75	Inoculation of 1 shell vial (1 cm²) for the study of generation time, 9 shell vials for stock preparation (frozen), and 5 25-cm² flasks for the study of growth conditions
95	Passage of the shell vial on a 25-cm² flask
285	Generation of 3750 cm² of infected tissue from the original shell vial passaged on day 30 (calculated doubling time, 18 days)

Transmission Electron Microscopy

On day 105, about 1000 infected cells from a second-passage flask were prepared for examination with a transmission electron microscope (model 1220, Jeol, Croissy sur Seine, France) as described previously.[18]

Immunofluorescence Staining

On day 105 the monolayer from one shell vial was examined by direct immunofluorescence as previously described,[16,19] with the use of the patient's serum as the primary antibody. Coverslips were examined with a laser scanning confocal fluorescence microscope (model DMIRBE, Leica, Wetzlar, Germany) equipped with an oil-immersion lens (100×). To evaluate our serologic methods, we also analyzed nine serum samples from patients with proved Whipple's disease. The serum sample from our patient, one from a patient with endocarditis due to Whipple's disease as proved by PCR testing,[20] and seven from patients with histologically proved Whipple's disease (in two of whom *Tropheryma whippelii* DNA was detected in duodenal tissue by PCR assay)[1] were tested. The group of seven patients were considered to have classic Whipple's disease, to distinguish them from the two patients with endocarditis due to Whipple's disease. Forty-one serum samples were used as negative controls: 11 were obtained from patients with autoimmune diseases, 10 were obtained from patients with endocarditis due to *Coxiella burnetii* (5 patients) and to *Bartonella quintana* and *B. henselae* (5 patients), and 20 were obtained from healthy blood donors.

We devised a procedure that uses eight-well Lab-Tek chamber slides (Nunc, Naperville, Ill.) and allows 12 slides to be prepared at a time. The supernatant was removed from a 150-cm² cell-culture flask containing approximately 10,000 HEL cells and in which infection was present in all fields (+++) by semiquantitative count.

Four milliliters of 0.25 percent trypsin (GIBCO, Grand Island, N.Y.) was added to the cell monolayer, and the culture was incubated at 37°C for about 10 minutes until the cells became separated from the bottom of the flask. The cells were then resuspended in 35 ml of fresh medium, and 350 μl of suspension was added to each well of the Lab-Tek chamber slides. The slides were incubated for 12 hours at 37°C in the presence of 5 percent carbon dioxide so that the cells could adhere to the glass slide. The serum samples were diluted in phosphate-buffered saline (1:25, 1:50, and 1:100) that contained 3 percent nonfat dry milk, and the titers of IgG and IgM were determined. For patients with IgM titers of 1:25, the serum samples were diluted from 1:50 to 1:400. To remove IgG, rheumatoid factor adsorbant (RF-absorbant, Behringwerke, Marburg, Germany) was added before the determination of IgM, according to the manufacturer's instructions. The culture medium was removed, the cells were fixed with methanol, and then the wells were washed twice with phosphate-buffered saline. Next, 100-μl samples of each serum dilution were added to the wells, and the chamber slides were incubated in a moist chamber at 37°C for 30 minutes. The slides were washed three times with phosphate-buffered saline and then incubated for 30 minutes at 37°C with 100 μl of goat antihuman IgG (Fluoline G, BioMérieux) or IgM (Fluoline M, BioMérieux) at a dilution of 1:300 in phosphate-buffered saline. The slides were washed three times with phosphate-buffered saline, the plastic upper structures mounted on the slides were removed, and the slides were mounted in phosphate-buffered glycerol medium (pH 8) and examined at a magnification of 400 with an epifluorescence microscope (Zeiss, Thornwood, N.Y.).

Production and Characterization of Mouse Polyclonal Antibodies

Immunocompetent BALB/c mice that were six to eight weeks of age were inoculated subcutaneously with 0.5 ml of a solution containing 10^6 of the bacteria obtained from the supernatant of infected cells mixed with 0.5 ml of Freund's complete adjuvant. The mice were inoculated on days 0, 10, 20, and 30. On day 40, the mice were killed and the antibody titers were measured by microimmunofluorescence testing. Before further use, the serum samples were diluted 1:50 and adsorbed on HEL cells to remove nonspecific anti-cell antibodies.

Amplification and Sequencing of the 16S Ribosomal RNA Gene

Bacterial DNA was extracted from 500 μl of supernatant from a cell-culture flask by Qiagen columns (QIAmp tissue kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification with the broad-range 16S ribosomal RNA gene primers fD1 and rP2 and sequencing and purification of PCR products were performed as previously described.[21]

Statistical Analysis

We used Fisher's exact test for all statistical analyses. All P values are one-sided.

RESULTS

Gross pathological examination of the excised aortic valve revealed that the cusps were thickened, distorted, and fibrotic, with a large friable vegetation. Histologically, the aortic valve had organizing superficial platelet–fibrin thrombi on the cusps with focal calcific deposits and necrotic cellular debris. These vegetations were associated with extensive fibrosis and with acute and chronic inflammation. The granulation tissue beneath the surface of the cusp included a chronic inflammatory infiltrate with numerous foamy macrophages. PAS-positive bacilli were identified in coarse masses of rod-shaped bodies within the foamy macrophages, the hallmark of Whipple's disease.[22,23] However, no microorganisms were detected on staining with Giemsa, Brown and Hopps, Gomori–Grocott, or Warthin–Starry stains. The chronology of isolation of the bacterium for Whipple's disease is summarized in Table 1. A cytopathic effect and microorganisms were not detected until day 65 after inoculation. Using an inverted microscope, we identified small, coarse, dark inclusions and large, coarse, round structures within cells on day 72 (Fig. 1). Gimenez staining of the supernatant after centrifugation revealed several slender pink bacilli. The majority were intracellular, and the intracellular bacilli were shorter than those outside the cells. However, most of these bacilli were poorly stained or not stained by the Gimenez stain and appeared pale blue. Numerous bacilli were also revealed by Gram's staining. Most were gram-positive, but several were only partially purple or gram-negative. On Ziehl–Neelsen staining, these bacilli were not acid-fast. More bacilli were PAS-positive than were positive for the Gimenez or Gram's stains. The cells were filled with coarse, PAS-positive conglomerates and short, slender, PAS-positive rods.

Figure 1. Large, Coarse, Round Structures (Arrows) within HEL Cells in a Six-Week-Old Culture of the Whipple's Disease Bacterium.

An inverted microscope was used. The bar represents 30 μm.

Amplification and sequencing of the 16S ribosomal RNA gene of the isolate produced a segment of 1450 bp. We compared the sequence with DNA-sequence data bases (Blast, version 2.0, National Center for Biotechnology and Information, Bethesda, Md.) and found that it was 99.9 percent homologous to the 16S ribosomal RNA sequence of *T. whippelii* (European Molecular Biology Laboratory accession number, X99636).

All subculture procedures used for the propagation of the isolate were effective, since in all cases, the isolate was recovered after 30 days of subculture. However, the most effective procedures were inoculation of supernatant onto fresh cell monolayers, as

was done in the case of flask A, or the duplication of infected cells, as was done in the case of flask D2. In those cases, the cultures were evaluated semiquantitatively after 30 days of subculture, and bacilli were easily detected, but not in all fields. The results of attempts to subculture the bacilli on MRC 5 cells were similar. All attempts at subculture on axenic medium were unsuccessful. At each stage of the propagation procedure, 500-μl samples of the cultures were tested by PCR and the bacterium of Whipple's disease was confirmed to be present in the culture.

Immunofluorescence staining demonstrated that staining with PAS and other stains underevaluated the extent of cell infection. Immunofluorescence examination of shell-vial coverslips after 30 days of subculture showed that all cells contained large amounts of the isolated antigen. The intracellular location of the bacilli was confirmed by confocal microscopy (Fig. 2A). Several bacteria were seen that resembled the short, slender rods observed on PAS staining. Nevertheless, most immunopositive material was found in larger inclusions, where individual bacteria were not seen. No immunopositive material was detected within the nuclei. Large numbers of bacteria were found by acridine orange staining of the cell-culture supernatant (Fig. 2B). Transmission electron microscopy confirmed that the PAS-positive inclusions and immunopositive material corresponded to intact and degenerating bacteria. Dividing cells were observed. The cell wall included a structure whose presence was consistent with previous descriptions of the bacterium of Whipple's disease.[24] The plasma membrane was surrounded by a thin, homogeneous wall, which was itself surrounded by a plasma-membrane–like structure, giving a trilamellar appearance (Fig. 3).

IgG antibodies against the bacillus were detected in most serum samples, including those from the control subjects. Cutoff values were selected after the results were known. When a cutoff value of 1:100 was selected, samples from all nine patients with confirmed Whipple's disease (endocarditis or classic) were positive, as compared with samples from 29 of 40 controls ($P=0.08$) (Table 2). The presence of IgM antibodies was more specific to patients with Whipple's disease. When a cutoff value of 1:50 was selected, 7 of 9 patients with Whipple's disease had positive results, as compared with 3 of 40 control subjects ($P<0.001$). Both patients with Whipple's disease endocarditis had a positive IgM antibody titer, as compared with none of the 10 control subjects with endocarditis from other causes ($P=0.015$). Five of 7 patients with classic Whipple's disease had a positive IgM antibody titer, as compared with 2 of 10 control subjects with autoimmune diseases ($P=0.052$). The IgM antibody titer was 1:400 or more in three of seven patients with classic Whipple's disease and in both patients with Whipple's disease endocarditis, but in none of the patients without Whipple's disease. The serologic results for one control subject with autoimmune disease who had antineutrophil cytoplasmic autoantibodies were uninterpretable because of the presence of diffuse immunofluorescence.

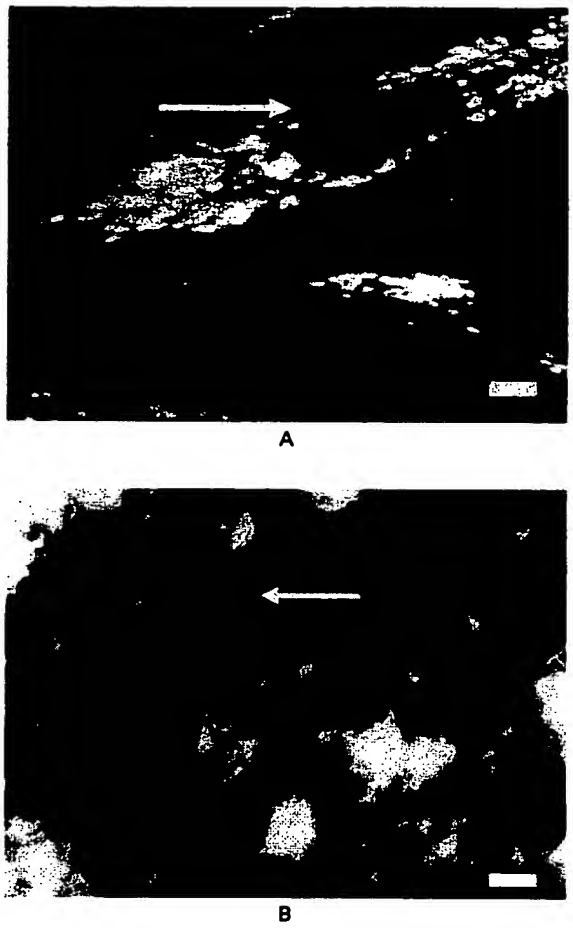

Figure 2. Whipple's Disease Bacterium.
For immunofluorescence staining of the bacteria (arrow) in infected HEL cells, the patient's serum was used as the primary antibody (Panel A). Specimens were examined with a confocal microscope. In Panel B, acridine orange staining reveals the numerous bacteria (arrow) in the supernatant of infected HEL cells. The bar in Panel A represents 6 μm, and the bar in panel B represents 15 μm.

Antibodies were produced in mice at high titers (1:1000) and were successfully used to detect the bacteria in the patient's excised tissue by immunochemistry.

DISCUSSION

Although Whipple's disease was identified nearly a century ago, the causative agent of this bacterial infection has not been successfully established in vitro. Even though no type strain of the bacterium is available, it is usually referred to by its provisional

Figure 3. Transmission Electron Micrograph Showing the Bacterium (Arrow) in Infected HEL Cells.
The bar represents 500 nm.

name, *T. whippelii*.[8,25] The molecular identification of this bacterium in biopsy specimens or peripheral-blood samples is the basis for the diagnosis.[9] However, this technique remains to be validated, and the diagnosis of Whipple's disease requires a biopsy specimen for either microscopical study or PCR analysis. Therefore, a serologic test could be highly useful, since the diagnosis could be made on the basis of a single blood sample. This is particularly important in patients with life-threatening complications such as endocarditis.

Unlike previous investigators who completed only two passages of the bacterium cultures of human macrophages,[12] we used a human fibroblast cell line with no specific culture conditions. We completed seven passages of our isolate and believe that the culture is now definitely established. By day 285 we had 120 heavily infected 150-cm² cell-culture flasks. The strain has been deposited in the French National Collection at the Pasteur Institute in Paris and is available. Since it requires 210 days to obtain 25 well-infected 150-cm² cell-culture flasks from a 1-cm² shell vial, the generation time (or doubling time) of the bacterium is about 18 days, which is similar to that for *Mycobacterium leprae* in animal models (12 days).[26] Patience has been a key to culturing new pathogens, as in the case of bartonella species,[27] which can take as long as 45 days to isolate, and *Helicobacter pylori*, for which a prolonged incubation is also necessary.[28] One other difficulty is the staining of bacteria in cells. PAS is useful but fails to detect all bacteria, unlike immunofluorescence of the tested strain. Acridine orange was the most useful stain. However, mouse-specific antibodies may be very useful for this purpose.

The successful isolation of intracellular bacteria is partly based on two critical points. First, the ratio of bacteria to cells should be as high as possible. Second, centrifugation, which was shown to enhance the adhesion of other intracellular bacteria to the cells,[29] probably favored isolation of the bacillus.

Table 2. Results of Indirect Immunofluorescence Assay of Serum Samples from Patients with Whipple's Disease and Control Subjects.

Group	No. of Subjects	IgG Antibody Titer ≥1:100	IgM Antibody Titer ≥1:50
		no. of subjects	
Patients with Whipple's disease	9	9*	7†
Whipple's disease endocarditis	2	2	2‡
Classic Whipple's disease	7	7	5§
Control subjects¶	40	29	3
Endocarditis	10	9	0
Autoimmune disease	10	9	2

*P=0.08 for the comparison with all control subjects.

†P<0.001 for the comparison with all control subjects.

‡P=0.015 for the comparison with control subjects with endocarditis.

§P=0.052 for the comparison with control subjects with autoimmune disease.

¶Twenty control subjects were healthy blood donors. The assay results for one control subject with autoimmune disease were uninterpretable and were therefore excluded from the analysis.

We used our serologic method in different groups of patients. IgG antibodies were detected more frequently in patients with Whipple's disease, but they were present at a titer of 1:100 or more in 29 of 40 control subjects and therefore are not suitable for diagnostic purposes. We do not know whether this high level of IgG results from previous contact with the bacterium or from a cross-reaction with other bacteria. However, the fact that all patients with Whipple's disease had antibodies to the isolated bacterium provides support for the idea that it has a causative role in the disease.

The presence of IgM antibodies at a titer of at least 1:50 was significantly associated with Whipple's disease overall and with Whipple's disease endocarditis. For the comparison with patients with classic Whipple's disease, we chose patients with autoimmune disease as the control group, a controversial choice, because these patients frequently have false positive serologic reactions. The fact that we removed rheumatoid factor from the serum samples may have controlled for the rare false positive result. In fact, in one patient, the presence of antineutrophil cytoplasmic autoantibodies made it impossible to interpret the results. However, the difference between the two groups was of borderline significance (P=0.052). The serologic data are preliminary, and it would be interesting to learn whether patients have higher titers of IgM antibodies early in the course of the disease.

Our results are encouraging, and the establishment

of the strain will enable researchers to define the role of this microorganism in several clinical syndromes. It may also be useful in developing an animal model of Whipple's disease as well as for antibiotic-susceptibility testing. Finally, the purification of the strain will clear the way for genetic studies, so that specific gene sequences can be obtained, in contrast to the current ones, which are based on universal primers.

We are indebted to Dr. D. Ross and Dr. R. Baskett of the Division of Cardiovascular Surgery at Dalhousie University, to Dr. A. Raza of the Department of Pathology at Dalhousie University, and to C. Capo for the confocal-microscopy images.

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Correction to Ganzini et al., *N Engl J Med* 342 (8) 557-563 February 24, 2000.

Correction to Raoult et al., *N Engl J Med* 342 (9) 620-625 March 2, 2000.

CORRECTION

◄ Previous **Volume 342:1538 May 18, 2000 Number 20** Next ►

Corrections

Physicians' Experiences with the Oregon Death with Dignity Act . On page 560, in line 9 of the right-hand column, the statement should read, "physicians reported that 3 of the 28 changed their minds about obtaining a prescription for a lethal medication as a consequence of mental health intervention," not "3 of the 28 changed their minds about obtaining a prescription for a lethal medication," as printed. On page 562, the sentence that begins on line 16 of the left-hand column should have read, "In 27 cases the physicians had met this requirement by the time they completed the questionnaire," not "Twenty-seven of the physicians had met this requirement by the time they completed the questionnaire," as printed.

Cultivation of the Bacillus of Whipple's Disease . On page 620, on line 5 of the abstract and line 19 of the right-hand column, specimens from the *aortic* valve were obtained, not the *mitral* valve, as printed.

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Didier RAOULT et al.

Application No.: 09/936,921

Filed: September 24, 2001

For: DIAGNOSIS OF WHIPPLE'S DISEASE

Group Art Unit: 1645

Examiner: P. Baskar

Docket No.: 110530

DECLARATION UNDER 37 C.F.R. §1.132

I, M. Drancourt, a citizen of France, hereby declare and state:

a. I have a Ph. D. in Cell Biology and microbiology , which was conferred upon me by Université Aix-Marseille II in Marseille, France in 1988.

b. I have been employed by Université de la Méditérannée since 1990 and I have had a total of 20 years of work and research experience in Medical Microbiology.

c. I am a member of Société Française de Microbiologie.

d. My publications include the following works in this field: Drancourt, "*Tropheryma whippelii,* pathogène émergent à culture intracellulaire responsable de la maladie de Whipple". La Presse Médicale (1999; 28:435-439) (hereinafter "my 1999 Presse Medicale article"); Drancourt M, Raoult D, Lepidi H, Fenollar F, Birg ML, Bodaghi B, Hoang PL, Lelievre JD. Culture of *Tropheryma whippelii* from the vitreous fluid of a patient presenting with unilateral uveitis. Ann Intern Med. (2003; 139:1046-7); Raoult D, Ogata H, Audic S, Robert C, Suhre K, Drancourt M, Claverie JM. *Tropheryma whipplei* Twist: a human pathogenic Actinobacteria with a reduced genome. Genome Res. (2003; 13:1800-9); and Drancourt M, Carlioz A, Raoult D. rpoB sequence analysis of cultured *Tropheryma whippelii*. J Clin Microbiol. (2001; 39:2425-30).

e. I have reviewed the following article: Schoedon et al., "Deactivation of macrophages with interleukin-4 is the key to the isolation of *Tropheryma whippelii*". Journal of Infectious Diseases (1997; 176:672-677) (hereinafter "Schoedon"). In particular, I studied this article in connection with the preparation of my 1999 Presse Medicale article, which is identified above. In addition, I have subsequently reviewed this article in the preparation of this Declaration.

f. I have further reviewed about all the State of the Art in the field of *Tropheryma whipplei* cultivation, including the publications by Pr Raoult's team and the patent application WO0058440 corresponding to the above-identified U.S. patent application, and inter alia the following scientific publications in the preparation of this Declaration:

- Hinrikson et al. "Detection of three different types of Tropheryma whippelii" in International Journal of Systematic Bacteriology (1999; 49:1701-1706) (hereinafter "Hinrikson"),

- the abstract by Muller et al. "Cultivation of T. whippelii" in Gastroenterology vol.116, n°4, part 2, April 1999, page A910, Abstract XP002123745 (hereinafter "Muller"),

- Maiwald et al. "Cultivation of Tropheryma Whipplei from Cerebrospinal Fluid" Journal of Infectious Disease (2003; 188:801-808) (hereinafter "Maiwald"), and

- Bentley et al. "Sequencing and analysis of the genome of the Whipple's disease bacterium Tropheryma whipplei" The Lancet (2003; 361:637-644) (hereinafter "Bentley").

g. I have further reviewed in the preparation of this Declaration:

- the attached letter by Schoedon dated 22 December 1998 sent to myself, and

- the laboratory books of Professor Raoult's team concerning culture of the *Tropheryma whipplei*.

I can now declare and state:

1. Schoedon and Muller report studies carried out on cultures of bacteria they reported as *Tropheryma whipplei* in human blood monocytes. They claimed to have isolated 2 such isolates. Such a culture was abandoned as recognized by Pr Schoedon in the above mentioned letter. Also, none of the 2 isolates has been made available in a strain deposit collection contrary to international code in microbiology, which asks that new isolates be deposited in public collection while it has long been sought to obtain such culture by the scientists in this area.

2. Schoedon and Muller have never more published since these first articles in 1997 and 1999 on that subject while their findings was of very great importance and had been long sought in this area. (see Maiwald, page 801: "*Cultivation of this bacterium has therefore been a goal of clinicians and microbiologists for several decades*".)

3. I can testify that Pr Raoult's team has attempted to reproduce the results reported by Schoedon and Muller in following their experimental teaching and these attempts were unsuccessful.

4. Other teams involved in this technical field have also unsuccessfully attempted to confirm the results of Schoedon. In particular, as described in Maiwald, the finding by Schoedon of the propagation of bacteria could not be confirmed in subsequent studies. Maiwald, p. 802, col. 1, lines 4-9: "*..investigators inoculated interleukin-4-deactivated macrophages with heart-valve tissue affected by WD and reported propagations [8]. However, this finding could not be confirmed in subsequent studies [9]*" (Zaaijer et al.), and p. 805, col. 2, lines 2-5: "*A previous report [8], describing the growth of T. whipplei in interleukin-4-deactivated macrophages, has not been confirmed, either by us (M.M. and D.A.R., unpublished results) or by other investigators [9]*". This was further confirmed by two of the authors of Schoedon,

Martin Altwegg and Fabrizio Dutly, who indicated in an article published about two years after Schoedon (Hinrikson), that the relationship between clinical manifestations of Whipple's disease and different infecting strains of *Tropheryma whippeli* has not been studied "*mainly because of the absence of reliable cultures* (Schoedon *et al.*, 1997)." Hinrikson, p. 1705, col. 1, discussion. In fact, the summary of this article on page 1701 refers to *Tropheryma whippelii* as "*the uncultivated causative agent of Whipple's disease*" (emphasis added). In addition, the article indicates on page 1706 that certain studies are "*not feasible due to the fact that 'T. whippelii' has not yet been cultured on artificial media*". See also Raoult et al., The New England Journal of Medicine, Vol. 342, No. 9, pp. 620-625 (March 2, 2000), which indicates that the isolate described in Schoedon "*could not be subcultured*" (p. 620, col. 2).

5. Muller purports to describe the cultivation of *Tropheryma whippelii* in peripheral blood mononuclear cells (PMNC) treated with IL-4 and the cocultivation of *Tropheryma whippelii* in PMNC with macrophages and with the monocytic cell line U937, both deactivated by IL-4 pretreatment. The abstract indicates that its results "*were positive suggesting true bacterial growth in those cells*" and that treatment with IL-4 "*seems to induce replication of T. whippelii*" (emphasis added). Based on use of the terms "*suggesting*" and "*seems*," it is clear that the authors could not conclusively say that the bacteria had reproducibly multiplied in this culture. In addition, it is noted that this abstract has never been followed by a corresponding scientific publication.

6. By contrast the successful culture of *Tropheryma whipplei* according to the culture conditions taught by Raoult et al. have been confirmed by Pr Relman's team (see Maiwald and Bentley).

7. With the requirements in force today for the identification of bacteria by molecular biology, the results published by Schoedon would not be accepted as a definite

evidence that they have successfully cultivated the *Tropheryma whipplei* bacterium. Indeed, in their article the identification of the bacterium is based on a PCR with primers drawn from the 16S r DNA of the *Tropheryma whipplei* bacterium, which are universal primers and not considered as sufficiently specific of any bacterium. As acknowledged by Hinrikson, Dutly and Altwegg in their article of 1999 (Hinrikson, page 1701 column 2: "Several diagnostic *T. whippelii*-PCRs that target parts of the 16S rDNA have been established (Relman *et al.* 1992; . . Altwegg *et al.* 1996. . .). However, such systems may not discriminate between closely related species..."). Besides the PCR products obtained by Schoedon have not been sequenced, as now required for reliable identification of a new bacterium, but they have only be subjected to Southern blotting using an oligonucleotide of the 16S rRNA gene. The primers used by Schoedon were published by Relman (reference 4 of Schoedon), Maiwald (reference 13 of Schoedon) and Altwegg (reference 12 of Schoedon) and these authors have stated that they couldn't reproduce the experiments of Schoedon (see Maiwald and Hinrikson).

8. The kinetics results reported by Schoedon are not compatible with the more recent data concerning this bacterium. Indeed, reported kinetics of growth such as in Figure 4 and Figure 5 of Schoedon are absolutely not compatible with what was later known about the growth kinetics of *Tropheryma whipplei*. In the histogram of figure 4 as well as in the photographs of figure 5, they report passing from about 15-20% of cells infected by the bacteria to more than 50% in 48 hr. Such kinetics implies a doubling time of the bacteria lower than 24 hr. Such result is an extraordinarily rapid growth while *Tropheryma whipplei* is on the contrary particularly slow in growth with a doubling time of 18 days. At the time, it was not known that the doubling time of the *Tropheryma whipplei* bacterium was of 18 days.

9. It is likely that an organism mimicking some of the *Tropheryma whipplei* characteristics (intracellular growth, bacillary morphology, periodic acid-schiff positivity, reactivity with "TW-1/TW-3 or TW-1/TW-2" PCR primers) contaminated the Schoedon culture leading to the credence that *Tropheryma whipplei* had been isolated and cultured.

10. Another reason for the positive results reported by Schoedon could be that the initial inoculum they used was exceptionally high. This could be the possible reason why Schoedon was capable of detecting bacteria after 8-10 days of incubation followed by a passage and a further incubation of 10 days. However such exceptional circumstances, if any, in no way could prove that the *Tropheryma whipplei* bacterium was established in culture. Indeed, the man skilled in the art knows that to establish an intracellular bacterium in culture by using ex-vivo cells as did Schoedon, you can't obtain multiplication of the bacteria if the incubation period between two passages is lower than the doubling time of the bacterium. Under such conditions, you obtain a dilution of the bacterium as the ratio bacteria/cells numbers necessarily decreases and you will not be able to detect any bacterium after 3 or 4 passages, depending on the amount of bacteria in the initial inoculum. This could have been the reason why Schoedon did not report detection of bacteria after more than 4 passages under the conditions he taught, namely with passages after only 8-10 days of incubation and therefore was not able to depose the isolate in a bacterial collection and was led to "abandon the culture".For one of ordinary skill in the art, it is implicit in the phrases "established in culture" and "multiplies over time "that the ratio of bacteria to cells increases. In addition, it is also implicit in these phrases that the culture can be maintained over time , i.e. indefinitely.

11. In any event, it is practically and biologically impossible to establish in culture this *Tropheryma whipplei* bacterium for diagnostic purpose, in Il4 deactivated human monocytes as reported by Schoedon because the mean lifetime of monocytes is only 30 days, and human monocytes can't be furnished in sufficient amount to establish a bacterium in culture for biological diagnostic purpose. These cells are the more so unsuitable in view of the doubling time of the bacterium as explained above.

12. My 1999 Presse Medicale review article provided a summary of various articles concerning *Tropheryma whippelii*. It does not set forth the results of further experimentation. In particular, in my 1999 Presse Medicale article, I indicated that two strains of *Tropheryma whippelii* were isolated in a cell culture from two heart valves sampled from two different patients and that the strains were subsequently cultivated in a human line of monoblasts SigM5. In support of these statements, I referred to Schoedon. My statements do not reflect any experimentation that I conducted to confirm the accuracy or repeatability of the work described in Schoedon. Instead, this paper merely summarized the work of researchers in the field such as reported in the scientific literature, including Schoedon.

13. In view of all the above considerations, based on my own investigation and the more recent scientific publications by other investigators, it can be recognized that Raoult et al. (i.e., the inventors of the above-identified U.S. patent application) have been the first to teach the suitable conditions to establish the Tropheryma whipplei bacterium in culture as acknowledged in Maiwald (see the abstract: "*..many attempts have been made to cultivate this bacterium in vitro. It was eventually isolated, in 2000....*") and Bentley (see page 637 column 2: "*Isolation of the bacterium Tropheryma Whipplei was achieved in 2000, in a long term culture system with human fibroblasts, with a reported generation time of 18 days*" (Raoult et al 2000)).

14. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: August 18th 2005

M. Drancourt

Attachment:
December 22, 1998 Schoedon letter

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Zürich, 22. 12. 98

Dear Prof. Drancourt

We a very sorry to say that the isolate of *T. whippelii* is no longer available. Funding of our interdisciplinary research project on that topic was abandoned based upon the opinions of a board of international reviewers, claiming Whipple's disease beeing very rare in Switzerland and especially supply of material from additional patients would be very limited. Therefore we stopped culturing *Tropheryma* and turned to our original funded projects. We regret not being able to supply you with the requested material.

Sincerely yours

J. Schoedon

PD Dr Gabriele Schoedon

APPENDIX C - RELATED PROCEEDINGS APPENDIX

Copies of relevant decisions in the following related proceedings are attached:

NONE